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(54) Title: IMMUNOTHERAPEUTIC TREATMENT METHODOLOGY FOR PATIENTS AFFLICTED WITH SUPERFICIAL BLADDER CANCER WHO PREVIOUSLY FAILED AT LEAST ONE IMMUNOSTIMULATORY THERAPEUTIC TREATMENT REGIMEN

(57) Abstract: This present invention provides a broad immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer, where the patient has failed at least one immunostimulatory therapeutic treatment attempt previously. The invention also offers a first-time immunotherapeutic method for treating a living patient afflicted with upper urinary tract cancer, the ureter and renal pelvis regions of the body, an anatomic location where no effective cancer treatment has been available before. The methodology introduces an effective quantity of at least one viableMicobacterium species to the chosen anatomic site concurrently and in combination with at least one selective cytokine chosen from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18.

IMMUNOTHERAPEUTIC TREATMENT METHODOLOGY FOR PATIENTS AFFLICTED WITH SUPERFICIAL BLADDER CANCER WHO PREVIOUSLY FAILED AT LEAST ONE IMMUNOSTIMULATORY THERAPEUTIC TREATMENT REGIMEN

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FIELD OF THE INVENTION

The present invention is concerned with therapeutic treatments for bladder cancers; and is particularly directed to immunotherapeutic treatments for persons afflicted with superficial bladder cancers and upper urinary tract (ureters and renal pelvic region) cancers in the human body.

BACKGROUND OF THE INVENTION

Carcinoma of the bladder is the fourth leading solid malignant disease among men and the eighth among women in the United States [American Cancer Society, 1998, Cancer Facts and Figures]. The majority of bladder cancers (75 to 80%) are present initially as superficial tumors and over 90% are transitional cell in origin [Droller, M.J., "Transitional cell carcinoma: upper tracts and bladder", Campbell's Urology, W.B. Saunders, 1986]. Transitional cell carcinoma (TCC) also affects the upper urinary tract (ureter and renal pelvis) but with an overall incidence rate of 2-

4%. However, this rate rises to 10% in the setting of advanced bladder cancer; and rises to up to a 20% rate after successful BCG therapy of aggressive superficial bladder cancer.

Kinds of bladder cancers

Transitional cell carcinomas (the majority of cases present as superficial tumors) most often appear as papillary growths, but higher-grade lesions are often sessile and ulcerated. Grading is based on histologic architecture, size, pleomorphism, mitotic rate, and hyperchromatism. The frequency of cancer recurrence and progression is strongly correlated with grade, the grades being general categories of how well the tumor is differentiated (as shown by Table A). It will be recognized and appreciated that tumor grading is an approximation and estimate at best.

Table A: Grading of Tumors

Tumor Grade 1:	well differentiated cells
Tumor Grade 2:	moderately differentiated cells
Tumor Grade 3:	poorly differentiated cells
Tumor Grade 4:	relatively undifferentiated cells

Also, while progression may be noted in a few grade 1-2 cancers (5-15%), it is common with poorly differentiated and undifferentiated tumors (33-67%). Carcinoma in-situ is recognizable as a flat, nonpapillary, anaplastic epithelium and may occur focally or diffusely, but it is most often found in association with papillary bladder cancers. Its presence identifies a patient at increased risk of recurrence and progression. Adenocarcinomas and squamous cell cancers account

for approximately 2% and 7% (respectively) of all bladder cancers detected in the USA. The latter is often associated with schistosomiasis, vesical calculi, or chronic catheter use.

Bladder cancer staging is another scheme for characterization of tumors. Bladder cancer staging is based on the extent of bladder wall penetration and the presence of either regional or distant metastases. The TNM classification of the American Joint Cancer Committee for bladder cancer staging is shown by Table B below. By definition and staging criterion, superficial bladder cancers include any of the T0, Tis, Ta and T1 stages.

Table B: TNM staging system for bladder cancer

T: Primary tumor

Tx	Cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in-situ (CIS)
Ta	Noninvasive papillary carcinoma
T1	Invasion into lamina propria
T2a	Invasion into superficial layer of muscularis propria
T2b	Invasion into deep layer of muscularis propria
T3a	Microscopic invasion through serosa into perivesical fat
T3b	Microscopic invasion into perivesical fat
T4a	Invasion into adjacent organs
T4b	Invasion into pelvic sidewall

N: Regional lymph nodes

Nx	Cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node ≤ 2 cm
N2	Metastasis in a single lymph node >2 cm and <5 cm or multiple nodes none >5 cm
N3	Metastasis in lymph node >5 cm

M: Distant metastasis

Mx	Cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

Bladder tumor progression and recurrence

The natural progression history of bladder cancer is based on two separate but related processes: tumor recurrence; and progression to higher stage disease. Both are related to tumor grade and stage. At initial presentation, approximately 70-80% of bladder cancers will be superficial: Ta, Tis, T1. Lymph node metastases and progression are uncommon in such patients when they are properly treated; and 10 year survival is excellent at 81%. Patients with superficial cancers (Ta, T1) are typically treated with complete transurethral resection and the selective use of intravesical therapy. The latter is used to prevent or to delay recurrence. Patients who present with large, high-grade, recurrent Ta lesions, those with T1 cancers, and those with carcinoma in-situ are deemed to be appropriate candidates for intravesical therapy. Patients with more invasive (T2, T3), but still localized, cancers are at risk of both nodal metastases and progression; and these persons require more aggressive surgery, irradiation, or the combination of chemotherapy and selective surgery or irradiation due to the much higher risk of tumor progression compared to patients with lower-stage lesions. Patients with evidence of lymph node or distant metastases typically undergo systemic chemotherapy initially.

Also, while most bladder tumors can be surgically resected through endoscopic techniques (CIS is not surgically accessible due to its diffuse surface-spreading behavior), tumors recur roughly 60% of the time and progression to life-threatening disease eventually occurs aggregately in 20-25% of cases. Among bladder tumors, aggressive and relatively non-aggressive subsets can be identified. Aggressive tumors come in three basic forms: (a) tumors that have invaded through the basement membrane (Stage T1); (b) tumors with poorly differentiated or undifferentiated cellular histology (grades 3 and 4); and (c) carcinoma in-situ (CIS). Progression rates for these three categories average 20-30%, 40-50%, and 50-80% respectively. Once tumor progression has occurred, most patients require radical surgery to control the disease. Relatively non-aggressive bladder tumors are confined to the mucosa (Stage Ta) and well-moderately differentiated (Grade 1-2); but other characteristics such as size, multiplicity, and recurrence rate may also lead

to progression in 5-15% of these cases. Recurrence rates are similar among both aggressive and non-aggressive subtypes.

Intravesical therapeutic treatment approaches

Immunotherapeutic or chemotherapeutic agents can be and most commonly are delivered directly into the bladder by a urethral catheter. These agents can be used to eradicate existing disease or to reduce the likelihood of recurrence in those who have undergone complete transurethral resection. Such therapy is more effective in the latter situation. Most of the agents are administered weekly for 6-12 weeks. The use of maintenance therapy after the initial induction regimen may be beneficial in some cases. Efficacy may be increased by prolonging contact time. The common agents include thiotepa, mitomycin, doxorubicin, and BCG, the last of these being the most effective agent when compared with the others. The known side effects of intravesical chemotherapy include irritative voiding symptoms and hemorrhagic cystitis, but systemic effects are rare.

BCG Intravesical Treatments

Initial intravesical treatment with the live attenuated vaccine strain of *Mycobacterium* bovis-Bacillus Calmette-Guerin (BCG) is believed to activate the cellular immune response, which is impaired in urinary bladder carcinoma as reflected by a diminished blastogenic response and decreased production of interleukin-2 (IL-2) and interferon-gamma (IFN-γ). Following local administration of BCG, a persistent increase in mononuclear cells, predominantly CD4-positive helper T cells, has been observed in the bladder, leading to an elevated CD4/CD8 ratio. This cellular increase, however, is independent of the clinical response to BCG treatment. A summary of the cytokines, immune cells, and other molecules detected is provided by Table C.

Table C:ϕ Cytokines, immune cells and other molecules detected locally after BCG immunotherapy of bladder cancer

Cytokine	Function	Possible sources
IL-1	Co-stimulation of T lymphocytes; induction of adhesion molecules	Macrophage, PMN, urothelium*
IL-2	T-cell proliferation; enhancement of cytotoxicity; rescue from anergy and apoptosis of T cells and neutrophils	T cell
IL-6	Co-stimulation, upregulation of IL-2 receptor on T cells; inhibition or stimulation of tumor cell growth; induction of IL-1, IL-8, TNF	T cell, macrophage, urothelium, PMN
IL-8	Chemotaxis of T lymphocytes and neutrophils	T cell, urothelium, macrophage, PMN
IL-10	TH2 polariser; suppresses TNF, ICAM-1, B7 and MHC induction	T cell, macrophage, urothelium?
IL-12	TH1 polariser; induction of T cell cytotoxicity, rescue from anergy and costimuation	T cell, macrophage
IL-18	Enhances γ interferon production	Urothelium?, macrophage
GM-CSF	Costimulation and enhancement of T cell cytotoxicity; rescue of macrophages from apoptosis; maturation of dendritic cells†	Macrophage, T cell, urothelium, PMN
interferon-γ	Stimulates IL-12 production; anti-angiogenic‡, induces ICAM-1, B7, MHC I and II, CD40, FAS; cytostatic and cytotoxic	T cell, macrophage
TNF	Co-stimulation and enhancement of T-cell cytotoxicity; cytostatic and cytotoxic; induces MHC class I and II, ICAM-1, FAS, IL-6, IL-8; anti angiogenic‡	T cell, macrophage, PMN, urothelium

Immune cells	Function	Notes
Cytotoxic T lymphocyte	Induction of apoptosis and tumor cell lysis	Unclear whether they are tumor specific
T-helper lymphocyte	Production of cytokines (IL-2, interferon γ, TNF, IL-12); bystander lysis of tumor cells; help to BCG-activated killer cells	Unclear whether they are tumor specific In-vitro phenomenon
BCG-activated killer cells	Tumor specific destruction	Determine genetic resistance to therapy through *Nramp1* alleles
Macrophage	Antigen presentation§; bystander tumor destruction; help to BCG-activated killer cells	First and most abundant infiltrate;suppress/augment side-effects?
Neutrophil	Cytokine production (e.g., IL-8, IL-12, GM CSF, interferon alpha, MIPα and β, TNF, IP-10)	

Other key molecules	Receptor	Function
MHC class I	CD8	Antigen presentation to cytotoxic T cells
MHC class II	CD4	Antigen presentation to helper T cells
ICAM-1	LFA-1	Adhesion anchor and co-stimulation for antigen presentation
FAS	FAS-ligand	Delivery of cytotoxic signal to target cell-inducing apoptosis
CD40	CD40-ligand	Co-stimulation for antigen presentation, growth regulation

Notes:
FAS=TNF-like receptor, ICAM=intercellular adhesion molecule, IL=interleukin, IP=interferon gamma inducible protein, LFA=leukocyte function-associated molecule, MHC=major histocompatility complex, MIF=migration inhibition factor, PMN=polymorphonuclear leukocyte, TNF=tumor necrosis factor.
*Includes both benign and malignant epithelium. †Presently there is little if any evidence of involvement of dendritic cells in BCG immunotherapy. ‡IP-10 also exerts potent anti-angiostatic effects. §Providing there is tumor-specific response; however, note that bladder cancer cells can also present antigen to T lymphocytes.

ϕ Adopted from: Alexandroff et al., Lancet 353: 1689-1694 (1999) at p. 1691.

It is recognized, however, that the primary therapeutic effect of BCG intravesical treatment is inducing the local production of cytokines in the bladder, as characteristically shown by the appearance of IL-1, IL-2, and TNF-α in the urine of the patient. See for example: Arad et al., Cell Immunol. 160: 240 (1995); Boccafoschi et al., Eur. Urol. 21: 304 (1992); Bohle et al., J. Urol. 144: 53 (1991); Bohle et al., Dev. Biol. Stand. 77: 199 (1992); Bretton et al., J. Urol. 143: 710 (1990); Brosman, J. Urol. 128: 27 (1982); Coplen et al., J. Urol. 144: 652 (1990); DeJong et al., Cancer Immunol. 31: 182 (1990); Fleischman et al., Cancer 64: 1447 (1989); Kaempfer et al., J. Clin. Oncology 14: 1778 (1996); Lamm et al., J. Urol. 124: 38 (1980); Ratliff et al., J. Urol. 150: 1018 (1993); Shapiro et al., J. Urol. 128: 891 (1982).

Intravesical BCG therapy is commonly applied as an adjuvant treatment to prophylax against tumor recurrence; and as a therapeutic treatment to eliminate residual small volume disease and CIS. Immunotherapy with BCG has consistently resulted in initial complete response rates of 55-65% for papillary tumors and 70-75% for CIS [Morales et al., J. Urol. 125: 649 (1981); Lamm et al., N. Eur. J. Med. 325: 1205 (1991)]. Long-term studies have documented benefits in terms of decreased recurrence rate, decreased progression rate, reduced cystectomy rate, and even improved survival [Herr et al., J. Urol. 135: 265 (1986)]. By contrast, intravesical therapy with cytotoxic chemotherapeutic agents provides an estimated benefit of between 7-16% without any documented evidence of prolonged efficacy [Herr et al., J. Urol. 138: 1363 (1987)]. The large preponderance of controlled trials randomizing patients with superficial bladder cancer to BCG treatment or chemotherapy have shown strong statistically significant responses that in the aggregate are roughly 3 fold greater for BCG [Traynelis et al., Urol. Annual. Vol. 8, 113 (1994)]. For these reasons, initial treatment with BCG is currently regarded as the most effective intravesical agent for the treatment of superficial transitional cell carcinoma (TCC) of the bladder.

Failures of BCG Intravesical Treatment

Unfortunately, it will be noted and appreciated, also, that up to 50% of patients fail an initial induction course of BCG treatment. Institution of a single re-induction course after an initial failure (i.e., BCG failure x 1) has been shown to decrease the total failure rate to 30-40%, but further re-treatments (i.e., BCG failure x 2, etc.) results in disappointing net responses of under 20% [Catalona et al., J Urol. 137: 220 (1987)]. Furthermore, the delay caused by additional minimally effective re-treatments is associated with a proportional increased risk of life-threatening disease advancement. Among initial BCG successes, 30-50% of patients gradually fail with recurrent bladder cancer within 5 years [Lamm et al., N. Engl. J. Med. 325: 1205 (1991)]. Long-term studies suggest a lasting benefit in as few as 20% of the original patient group by 10-15 years [Lamm et al., J. Urol. 157: 2134 (1997)].

There are likely several causes for failure of BCG treatment to durably eliminate bladder cancer recurrences. For instance, a clinically understaged bladder cancer thought superficial but actually muscle-invasive will not respond to BCG. Similarly, an excessive tumor load decreases responsiveness. Some cancers may even be intrinsically resistant to immunotherapy. However, a large percentage of patients treated with BCG fail because they do not make either an appropriate or sufficient local immune response in the bladder.

Alternative intravesical therapeutic treatments

A variety of alternative treatments have been investigated for patients failing BCG intravesical treatments. Merely illustrating some of these attempts is the data summarized by Table D below.

Table D:

Author (year)	Treatment	Patient Group	2 Yr NED	Number	Ref
Catalona ('87)	BCG 3rd course	Mixed	20%	6	1
Williams ('96)	Interferon-alpha	CIS only	12%	34	2
Malmstrom ('99)	Mitomycin C	Mixed	23%	19	3
Anthra ('99) FDA	Valrubicin	Mixed	<10%	90	4

NED = no evidence of disease
Mixed = mixed papillary stage Ta and T1 transitional cell carcinoma and/or CIS (Carcinoma in-Situ)

Refs:

1. Catalona et al., J. Urol. 137: 220-224 (1987);
2. Williams, et al., J. Urol. (part 2) 155: 494A, abstract 735 (1996);
3. Malmstrom et al., J. Urol. 161: 1124-1127 (1999);
4. Data on file, Anthra Pharmaceuticals Inc., FDA submission on 90 pt. Pivotal Trial.

As shown by the data of Table D, the various alternative treatments for patients failing BCG have been investigated but the clinical results are generally disappointing. Intravesical treatment with one of the most active intravesical chemotherapeutic agents, mitomycin C, for instance, resulted in only 23% of patients free of disease at 2 years after initial BCG failure. Durable 2-year disease-free rates of under 12% have been similarly reported for another intravesical chemotherapeutic agent, valrubicin, and for the immunomodulator interferon-alpha.

Treatment alternatives for patients with superficial TCC and failing BCG treatment(s) are limited and generally non-ideal. In addition to the agents mentioned in Table D, other investigational treatments including photodynamic therapy, Keyhole Limpet Hemocyanin [KLH] immunotherapy, and direct administration of single agent recombinant human cytokines such as Tumor Necrosis Factor-alpha [TNF-a] [Ernstoff et al., Surg. Rounds 417 (May, 1991); Glazier et al., J. Urol. 154: 66 (1995)], Interleukin-2 [IL-2] [Gomella et al., Cancer Biother. 8: 223 (1993); Boccon-Gibod et al., Proc. Assoc. Cancer Res. 35: 523 (1994); Otter et al., J. Urol. 159: 1183 (1998)], Interleukin-12 [IL-12] [Weiss et al., Proc. ASCO (April, 1999)], Interferon-gamma [IFN-γ] [Geboers et al., J. Urol. 137: 276A (1987)], Granulocyte Macrophage Colony Stimulating Factor (GMCSF) [Stravoraudi et al., J. Interferon Res. 19: 221 (1999)] and Interferon-beta [IFN-β] [Niijima et al., Cancer Immunol. Immunother. 30: 81 (1989)] have met with very limited success. For less aggressive bladder tumors, repetitive local surgery remains an option, but with over a 60% recurrence rate, the results are not satisfactory. For more aggressive bladder tumors (CIS, stage T1, grade 3) radical surgical removal of the bladder is usually recommended.

Of particular interest has been the use of a cytokine alone for intravesical therapy in treating superficial bladder cancers. Note that there have been no reports of the use of multiple cytokines for intravesical therapy. Representative of this alternative single cytokine approach has been the use of different individual interferons such as the α2b and β forms as immunotherapeutic agents [see for example: Belldegrun et al., J. Urol. 159: 1793-1801 (1998) and the references cited therein]. A useful summary of intravesical interferon therapy and its effects in

treating superficial bladder cancers and transitional cell carcinomas is given below by Table E, reproduced from the Belldegrun et al. 1998 publication.

Table E:

Intravesical interferon therapy in superficial bladder cancer

References*	No. Pts.	Ca Type	Drug	Dose	No. Response (%)	Duration (mos)
Oliver et al.	8	Papillary transitional cell Ca	Lymphoblastoid interferon	50 MU	3 Complete (37.5) 3 Partial (37.5)	No data
	8	Ca in-situ			0	
Torti et al.	16	Papillary transitional cell Ca	Recombinant interferon-α2b	50-1,000 MU	4 Complete (25)	17.5+ (mean)
	19	Ca in-situ/severe dysplasia		50-1,000 MU	6 Complete (32)	21+ (mean)
Glashan	38	Ca in-situ	Recombinant interferon-α2b	10 MU	2 Complete (5)	18
	47	Ca in-situ	Recombinant interferon-α2b	100 MU	20 Complete (43)	12+ (median)
Sarosdy et al.	33	Ca in-situ	Bropirimine	3.0 Gm.	20 Complete (61)	21+
Niijima	10	Papillary transitional cell Ca	Recombinant interferon-β	2-36 MU	0 Partial (0)	No data
	32	Papillary transitional cell Ca		3-36 MU/day	8 Partial (25)	No data
	9	Papillary transitional cell Ca		18 MU twice daily	6 Partial (67)	

* References cited are: Oliver et al., Brit. J. Cancer 53: 432 (1986); Torti et al., J. Clin. Oncol. 6: 476 (1988); Glashan, R.W., J. Urol. 144: 658 (1990); Sarosdy et al., Urology 48: 21 (1996); Niijima, T., Cancer Immunol. Immunother. 30: 81 (1989).

Table E (continued):

Intravesical interferon for transitional cell carcinoma prophylaxis

References+	No. Pts.	Drug	Dose	% Recurrence Rate	Time to Treatment Failure (mos.)	Median Followup (mos.)
Kostakopoulos et al.	30	Recombinant interferon-α2b	10 MU	37	Mean 11.2	12-28
Portillo et al.	78	Recombinant interferon-α2b	60 MU	54	–	43
Bartoletti et al.	19	Recombinant interferon-α2b	54 MU	21	Mean 9.4	23.8
Boccardo et al.	146	Recombinant interferon-α2b	50 MU	48	Median 21	14
	141	Mitomycin C	40 Mg.	37	Median 36	15.5
Hoeltl et al.	13	Recombinant interferon-α2b	10 MU	54	Mean 22.2	34.9
	11	Recombinant interferon-α2b	100 MU	46	Mean 22.4	37.7
	10	Ethoglucid	1.13 Gm.	60	Mean 21.8	37.2
Kälbe et al.	35	Recombinant interferon-α	10 MU	60	–	25
	32	BCG	120 Mg.	16	–	24

+ References cited are: Kostakopoulos et al., Eur. Urol. 18: 201 (1990); Portillo et al., Urology 49: 187 (1997); Bartoletti et al., Anticancer Res. 11: 2167 (1991); Boccardo et al., J. Clin. Oncol. 12: 7 (1994); Hoeltl et al., Brit. J. Urol. 68: 495 (1991); Kälbe et al., Urologe 33: 133 (1994).

Another alternative treatment approach has been a cytokine combination therapy, in a few instances utilizing BCG with a single cytokine. This idea has been investigated in a minimal way by the scientific community in the hope that the cytokine combination approach might exert antitumor activity. Merely illustrating the investigational effort is the published review of Belldegrum et al. [J. Urol. 159: 1793-1801 (1998) summarizing the case of recombinant interferons in combination with BCG. The information in that published summary is reproduced in part below as Table F.

Table F:
Interferon combination therapy for transitional cell carcinoma prophylaxis and treatment

Referencesϕ	No Pts.	Ca Type	Drug	Dose	% Recurrence Rate	Median Followup (mos.)
Engelmann et al.	22		Recombinant interferon-α2b	10 MU	18	6.2
	22		Recombinant interferon-α2b + mitomycin C	10 MU/20 mg.	0	6.2
	23		Mitomycin C	20 Mg.	22	6.2
Ferrari et al.	41		Recombinant interferon-α2b	50 MU	24	19
	44		Recombinant interferon-α2b + epirubicin	50 MU/80 mg.	16	19
Pavone-Macaluso and Serretta et al.	62		Recombinant interferon-α2b + epirubicin	5-10 MU/30-50 mg.	59	24
Stricker et al.	7	Ca in-situ	Recombinant interferon-α + BCG	10-100 MU/60 mg.	14	12
	5	Papillary transitional cell Ca			40	12
Bercovich et al.	18	Papillary transitional cell Ca	BCG	Not specified	28	24
	18		Low BCG + recombinant interferon-α		22	17
O'Donnell (personal communication)	5	Ca in-situ	Recombinant interferon-α2b + BCG	50 MU/27-81 mg.	20	3-12
	8	Papillary transitional cell Ca			25	3-12

ϕ References cited at: Engelmann et al., Anticancer Drugs (suppl). 3: 33 (1992); Ferrari et al., Anticancer Drugs (suppl.) 3: 25 (1992); Pavone-Macaluso et al., J. Chemother. 5: 207 (1993); Serretta et al., Urology 47: 647 (1996); Stricker et al., Urology 48: 957 (1996); Bercovich et al., Arch. Ital. Urol. Androl. 67: 257 (1995).

Finally, other investigators chose to focus their efforts upon ways and means for predicting in advance what an individual's clinical response to BCG might be prior to completion of treatment for superficial bladder carcinoma. Representative of this alternative approach is U.S. Patent No. 5,837,467 (and the references cited therein) which describes an in-vitro method which prior to the completion of therapeutic treatment calculates as a mathematical number the inducibility of the IL-2 gene (but not the IFN-gamma gene) using a sample of the patient's peripheral mononuclear blood cells as a predictor of his clinical response. The true value of this preductive in-vitro test procedure remains to be confirmed clinically by other investigators.

Summary of the present state of the art

The most efficacious adjuvant treatment of superficial bladder cancer was and remains today the instillation of live Mycobacterium bovis BCG (bacillus Calmette-Guerin) into the bladder. However, this BCG induction treatment fails in 30-40% of initial use cases and an additional 30-40% of initial responders relapse within 5 years. Furthermore, when this treatment fails, depending on the degree of bladder cancer aggressiveness, patients are faced with the prospects of repetitive surgical procedures; or complete bladder removal; or treatment with investigational intravesical agents; or even high dose chemotherapy and radiation therapy. Intravesical instillation of a cytokine immunostimulant such as interferon-alpha (IFN-a), while less effective than BCG, has been shown to induce complete responses in up to 40% of patients with superficial bladder cancer, although its durability is limited with most patients (50-65%) relapsing within one-two years. Combination therapy employing BCG and a cytokine has shown similar clinical results.

For the bladder cancer patient who has failed any of these immunostimulatory induction treatments (regardless of specifics), the prognosis is poor at best. For such failed immunostimulatory treatment patients, no efficacious followup or alternative immunotherapeutic treatment yet exists to date.

SUMMARY OF THE INVENTION

The present invention has multiple aspects and medical applications. A first aspect is provided by an immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer, said patient having failed at least one immunostimulatory therapeutic treatment attempt previously, said immunotherapeutic method comprising the steps of:

choosing the bladder for immunotreatment;

initiating at least one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one live Mycobacterium species to the bladder of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of at least one cytokine in an effective amount in the bladder of the patient, said cytokine being at least one selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and cytokine to act in combination in the bladder as an immunotherapeutic treatment for a preset period of time.

A second aspect of the present invention is an immunotherapeutic method for treating a living patient afflicted with upper urinary tract cancer, said immunotherapeutic method comprising the steps of:

choosing an anatomic site in the upper urinary tract region for immunotreatment;

initiating at least one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one live Mycobacterium species to the chosen anatomic site in the upper urinary tract of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of at least one cytokine in an effective amount at the chosen anatomic site in the upper urinary tract of the patient, said cytokine being at least one selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and cytokine to act in combination at the chosen anatomic site in the upper urinary tract as an immunotherapeutic treatment for a preset period of time.

A third aspect of the present invention provides an immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer, said patient having failed at least one cytokine-included treatment attempt previously, said immunotherapeutic method comprising the steps of:

identifying the cytokine administered previously to the patient in the failed immunostimulatory treatment;

choosing the bladder for immunotreatment;

initiating at least one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one live Mycobacterium species in the bladder of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of not less than one cytokine in an effective amount in the bladder of the patient, said at least one cytokine being different from that cytokine previously used in the prior failed treatment regimen for the patient, said at least one different cytokine being selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and at least one different cytokine to act in combination in the bladder as an immunotherapeutic treatment for a preset period of time.

A fourth aspect of the present methodology provides a primary immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer or upper urinary tract cancer, said patient not having received any immunostimulatory agents previously as a cancer treatment regimen, said immunotherapeutic method comprising the steps of:

choosing an anatomic site in the body of the patient for immunotreatment;

initiating not less than one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one live Mycobacterium species to the chosen anatomic site in the body of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain, a substantially non-pathogenic species Mycobacterium and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of not less than two cytokines in an effective amount at the chosen anatomic site in the body of the patient, said at least two different cytokines being selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and at least two different cytokines to act in combination at the chosen anatomic site in the body as an immunotherapeutic treatment for a preset period of time.

BRIEF DESCRIPTION OF THE FIGURES

The present methodology may be more easily and completely understood when taken in conjunction with the accompanying drawing, in which:

Fig. 1 is a depiction of the cytokine network evoked during conventional BCG immunotherapy of superficial bladder cancer depicting the role of proximal stimulating and inhibiting cytokines that lead to IFN-γ production. The density of the arrows roughly correlates to the importance of each individual cytokine.

Figs. 2A and 2B are graphs representing the kinetics of cytokine production from mouse splenocytes (2A) or human PBMCs (2B) after BCG stimulation. Values are expressed as a percentage of maximum measured amount. In both cases IFN-γ is among the latest cytokines expressed.

Figs. 3A and 3B show the dependence of IFN-γ induced by BCG-stimulation of mouse splenocytes (3A) or human PBMCs (3B) on proximal cytokines by selective antibody neutralization.

Fig. 4 is a graph showing that the multiple cytokines induced from mouse spleen cells after BCG stimulation can cooperate to recapitulate IFN-γ amplification. Purified recombinant murine cytokines were added to spleen cells in roughly the same concentrations as they are endogenous produced following BCG stimulation. *s depict the addition of IL-12 and/or IL-12 + IL-2 which are the most active agents in this system.

Fig. 5 is a graph illustrating the endogenous production of urinary cytokines (IFN-γ, IL-2, IL-10, and IL-12) in a patient receiving weekly intravesical BCG therapy for superficial bladder cancer.

Fig. 6 depicts the IFN-γ production of human PBMCs stimulated in-vitro with BCG plus a single exogenous cytokine as indicated. Based on IFN-γ production, cytokines were placed in functional groups including strong inhibitors, weak inhibitors, neutral, weak stimulators, and strong stimulators. The far right graph uses a y-axis scale roughly 300X that of the left graph. Controls included the

lectins phytohemagglutinin (PHA), concanavalin A (CON A) and phorbol myristate acetate (PMA)/Ionomycin (IM). Near identical results were obtained from PBMCs from a separate donor.

Figs. 7A and 7B demonstrate enhanced IFN-γ production from human PBMCs stimulated with the synergistic multicytokine combination of IL-2 + IL-12 + IFN-α with or without BCG. Fig. 7A shows how even extremely low concentrations of this multicytokine mixture retains strong stimulation potential with BCG. Fig. 7B shows how the BCG dose can be dramatically decreased 1000-fold in the presence of the multicytokine mixture and retain high activity.

Fig. 8 is a graph showing the induction of IFN-γ from murine splenocytes by different cytokine-secreting rBCGs. Enhanced activity is manifested for GMCSF rBCG, IL2 rBCG, and IFN-γ rBCG over a wide does range of BCG.

Fig. 9 illustrates the augmented IFN-γ stimulatory properties of rBCG secreting human rIL-2 or rIFN-α. Mixtures of the 2 rBCGs is further stimulatory and maintains high activity relative to control wild type (wt) BCG even when diluted down 1000-fold.

Fig. 10 is a graph showing the tumor burden in a mouse model bearing a syngeneic transplantable murine transitional cell carcinoma MB-49. Animals that were administered neutralizing antibody to IFN-γ showed enhanced tumor growth relative to control antibody IgG or phosphate buffered saline (PBS).

Fig. 11 indicates the value of urinary IFN-γ alone and the IFN-γ/IL-10 ratio in predicting cancer response (resp) or non-response(non resp) to conventional BCG monotherapy.

Fig. 12 is a graph showing the induction effects on IFN-γ of single cytokines, multiple cytokines, BCG and BCG plus single cytokines using the murine in-vitro splenocyte system.

Figs. 13A and 13B are graphs illustrating the inductive effect on IFN-γ of single cytokines, multiple cytokines, BCG and BCG plus single or multiple cytokines using the human in-vitro PBMC system. PBMCs from either immuno-protive (13A) or BCG-presensitized (13B) patients are responsive to BCG plus cytokine(s).

Figs. 14A and 14B demonstrate that IFN-α in the murine system behaves markedly differently than in the human system by decreasing IFN-γ production from BCG-stimulated splenocytes while increasing IL-10 production from the same.

Fig. 15 shows that BCG plus IL-12 administered directly in-vivo into the mouse bladder substantially increases the urinary production of IFN-γ.

Fig. 16 demonstrates that combination immunotherapy of BCG plus IL-12 reduces tumor growth more efficiently than either agent alone in the syngeneic, in-vivo, subcutaneous MB-49 mouse bladder cancer model.

Figs. 17A and 17B show the in-vitro PBMC and in-vivo urinary IFN-γ responses of patient MN who had originally failed prior BCG plus IFN-α intravesical therapy. In-vitro testing revealed combination BCG plus IL-12 would be most effective, an approach subsequently verified by in-vivo urinary cytokine monitoring.

Fig. 18 shows two graphs illustrating the induction of IFN-γ in human PBMCs by two different cytokine-secreting rBCGs.

Fig. 19 is a graph showing improved survival for bladder cancer bearing mice treated with rBCG expressing the interferon-inducible protein IP-10.

Fig. 20 shows the cancer free survival for 60 human bladder cancer patients after clinical treatment with BCG plus IFN-α in combination.

Fig. 21 shows the cancer free survival for a subgroup of 38 failed BCG bladder cancer patients after clinical treatment with BCG plus IFN-α in combination.

Figs. 22A and 22B are graphs showing the in-vitro PBMC response of patient MN to varying doses of BCG combined with IL-12 (22A) or varying doses of IL-12 combined with BCG (22B). Neither agent by itself is active while both together are profoundly stimulatory for IFN-γ production.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a broad immunotherapeutic methodology for treating different forms and types of cancer in the bladder and upper urinary tract of a living human. These treatable cancers are specifically the various tumors and neoplasms constituting superficial bladder cancers; and the different tumors and neoplasms found in the ureter and renal pelvis regions, the upper tract, of the human urinary system.

A particular benefit and advantage provided by the instant methodology is its focus and emphasis - a therapeutic treatment system and regimen for those bladder cancer patients who have already undergone one or more treatment attempts unsuccessfully; and presently have no medical recourse or course of treatment alternatives short of more radical methods for controlling, much less eliminating, their tumor. In this regard, the present innovations not only does provide substantial new treatment means for effective control and elimination of upper tract and superficial bladder cancers; this invention also provides a hope for the desperate and hopeless patient.

It is therefore deemed desirable to delineate and characterize the specific therapeutic goals as well as the kinds of cancer patients who will benefit most from the present methodology. Moreover, due to the unique theoretical model and mechanisms of stimulatory interactions upon which the present system is based, it is also valuable to review the underlying paradigm which lead to the instant immunotherapeutic treatment. The details and scheme of proper medical usage will be presented thereafter.

I. The Specific Therapeutic Goals And Patients

The present invention has three primary substantive objectives and goals. First, the invention is an immunotherapeutic treatment method specifically for patients with superficial bladder cancers (and transitional cell carcinomas in particular) who have undergone at least one immunostimulatory treatment therapeutic attempt (with or without prior surgery), but still have failed to achieve a

disease-free state of meaningful duration. Such persons collectively comprise "failed immunostimulatory therapeutic treatment patients"; and such failures of immunostimulatory therapeutic regimen include the individual categories of the failed BCG patients, the failed cytokine patients, and the failed BCG + cytokine patients, as each of these patient failure categories are defined and described hereinafter. Thus, this first therapeutic use and medical application is directed to those persons who have not been successfully treated for superficial bladder cancer using the conventionally known intravesical therapeutic treatment protocols and agents; and now are left without an effective treatment alternative aside from radical surgery or removal of the bladder.

The second major use and application of the invention is as the initial therapeutic treatment procedure for treating cancers, and transitional cell carcinomas in particular, of the upper tracts (anatomically, the ureter and renal pelvis) in the urinary system. The present treatment method is uniquely able to provide a regimen of immunotherapeutic induction treatment to the upper urinary tracts such that a remission occurs and a disease-free state eventually results in the ureter and renal pelvis areas. It will be noted and appreciated that cancers of the upper tracts regions are medically different and anatomically distinguished from superficial carcinomas of the bladder itself.

Third, the present treatment method can be effectively used as an initial induction treatment regimen for patients having bladder cancer, particularly transitional cell carcinomas, who have not previously had any immunostimulatory intravesical treatment or other immunotherapeutic regimen (surgery, chemotherapy, or irradiation treatments notwithstanding). Such patients are deemed collectively "immuno-pristine patients" (or "naive") in view of their lack of exposure to immunostimulatory agents. The present methodology relies upon a novel underlying mechanism of action model; and provides a meaningful alternative to the conventional modes of immunotherapy for bladder cancer patients as a first or initial regimen of therapeutic treatment.

Owing to the often ambiguous, misdescriptive, confusing and/or inappropriate names, titles, designations, terminology and jargon typically employed

by physicians, clinicians, research investigators, and laypersons to characterize and describe bladder cancers generally as well as the various therapeutic treatment protocols and procedures, a short listing of terms, designations and definitions is provided below which will be used consistently throughout this text for purposes of descriptive clarity and completeness. These terms and definitions are conventionally recognized and typically used by practitioners in the medical arts; and comply with the commonly understood denotative and connotative meanings associated with the word or term.

Definitions and Terminology

rBCG: Recombinant Mycobacteria species having an altered DNA content and presenting attenuated cell characteristics similar to M. bovis BCG which are suitable for immunotherapy purposes. Such recombinant Mycobacterial cells typically comprise at least one foreign or heterologous DNA sequence; and can include DNA coding for one or more biologically active cytokines which are expressed intracellularly and exported (secreted) extracellularly.

M. BCG or M. bovis BCG: Mycobacterium bovis Bacillus Calmette-Guerin species; a live attenuated bacterial strain.

BCG: A collective designation identifying either or both rBCG and/or M. bovis BCG.

Initial induction treatment: A first course of intravesical immunotherapy used as an initial therapeutic treatment for a previously immunologically untreated patient diagnosed with bladder cancer, the "naive" or immuno-pristine patient.

Second induction treatment: A second, followup, immunotherapeutic course of intravesical therapeutic treatment used as a second treatment regimen for a bladder cancer patient after the initial induction treatment has been completed or found to be unsuccessful or unsuitable.

Third induction treatment: A third, followup, immunotherapeutic course of intravesical therapeutic treatment used as a third treatment regimen for a bladder cancer patient after the initial and second induction treatments have been completed or found to be either unsuccessful or unsuitable.

Maintenance treatment: A course of intravesical therapeutic treatment instituted AFTER a disease-free status state has been achieved through one or more induction treatments. Accordingly, the purpose of maintenance treatment is to maintain the disease-free state by repetitive "boosters" of the therapeutic regimen.

rCytokine: An exogenous cytokine expressed in-situ by a recombinant Mycobacterial or other live species carrying a heterologous DNA segment coding for a cytokine of choice.

Cytokine: A discrete biologically active polypeptide typically expressed by blood mononuclear cells which can be either endogenous or exogenous, or a rCytokine.

Immuno-pristine patient: A bladder cancer patient who has not received any immunotherapy as a therapeutic regimen; but may have undergone chemotherapy, surgery or irradiation treatments. Also termed a "naive" patient.

BCG patient: A bladder cancer patient who has received at least one induction treatment with BCG as the therapeutic regimen.

Cytokine patient: A bladder cancer patient who has received at least one induction treatment with any cytokine or combination of cytokines without BCG as the therapeutic regimen.

BCG + cytokine patient: A bladder cancer patient who has received at least one induction treatment with BCG and a cytokine in combination as a therapeutic regimen.

It is presumed also that the reader has a basic knowledge of and familiarity with bladder cancers generally; the different locations, types and forms of tumors which can be found in the urinary system of humans; the conventional medical approaches (surgical, radiological, immunotherapeutic and chemotherapeutic) which have been reported and described in the medical and scientific publications; and the therapeutic compositions and clinical procedures typically used today for treatment of bladder cancers. The sum and substance of the state of this medical and therapeutic art has been presented and summarized in considerable detail previously herein as an aid and guide to the reader in order that he may better recognize and appreciate the broad scope of benefits and advantages provided by the present methodology.

In particular, it is essential to understand properly what the range and variety of different treatment failures comprise and include; and to recognize what such a treatment failure (frequently occurring in statistically large percentages) means for the bladder cancer patient as an individual fighting for his life against the cancer. Attention is therefore directed to Category Charts I, II, and III respectively as individually provided below.

Category Chart I: Failed BCG Patients*

Classes	Type(s)	BCG treatment regimen	Failures are characterized by
Refractory patients		Completed full course of primary treatment	Never achieved a disease-free state greater than 6 months' duration
Relapsed patients	early relapse	Completed full course of primary treatment; evidence of tumor regression or prophylactic effect; followed by a recurrence of tumor growth	Recurrence of tumor within 1 year's time post-treatment
	intermediate relapse		Recurrence of tumor between 1-2 years' time post-treatment
	late relapse		Recurrence of tumor after 2 years' time post-treatment
Intolerant patients		Unable to complete or tolerate one course of BCG immuno-therapy	No therapeutic effects; no remission of tumor

* Patients receiving at least an initial induction treatment with rBCG or M. bovis BCG.

Category Chart II: Failed Cytokine Patients**

Classes	Type(s)	Cytokine treatment regimen	Failures are characterized by
Refractory patients		Completed full course of primary treatment	Never achieved a disease-free state greater than 6 months' duration
Relapsed patients	early relapse	Completed full course of primary treatment; evidence of tumor regression or prophylactic effect; followed by a recurrence of tumor growth	Recurrence of tumor within 1 year's time post-treatment
	intermediate relapse		Recurrence of tumor between 1-2 years' time post-treatment
	late relapse		Recurrence of tumor after 2 years' time post-treatment
Intolerant patients		Unable to complete or tolerate one course of BCG immuno-therapy	No therapeutic effects; no remission of tumor

** Patients receiving at least an initial induction treatment with a cytokine which may have included among others IL-2, IL-12, TNF, GMCSF, IFN-α, β or γ or any combination thereof.

Category Chart III: Failed BCG + Cytokine Patientsϕ

Classes	Type(s)	BCG + a cytokine treatment regimen	Failures are characterized by
Refractory patients		Completed full course of primary treatment	Never achieved a disease-free state greater than 6 months' duration
Relapsed patients	early relapse	Completed full course of primary treatment; evidence of tumor regression or prophylactic effect; followed by a recurrence of tumor growth	Recurrence of tumor within 1 year's time post-treatment
	intermediate relapse		Recurrence of tumor between 1-2 years' time post-treatment
	late relapse		Recurrence of tumor after 2 years' time post-treatment
Intolerant patients		Unable to complete or tolerate one course of BCG immuno-therapy	No therapeutic effects; no remission of tumor

ϕ Patients receiving an initial induction treatment with BCG + a cytokine in combination, such as but not limited to BCG + IFNα or β, BCG + IL-2, BCG + IL-12.

As seen therein, the category of "failed BCG patients" includes many different kinds of patients suffering from superficial bladder carcinoma -- all of whom have received at least an initial induction treatment with BCG or M. bovis BCG as the immunotherapeutic regimen and have not responded well nor achieved a disease-free state of meaningful duration. Similarly, the separate and distinct category of "failed cytokine patients" includes all the different persons suffering from superficial bladder cancer who received an initial induction treatment using a cytokine as the mode of immunostimulation, but did not respond well nor reach a disease-free state of duration as a consequence of the therapeutic regimen. The third discrete category of "failed BCG + cytokine patients" is also recognized as constituting all the refractory, relapsed or intolerant patients who did not respond favorably and did not reach a disease-free state of meaningful duration after receiving the combination therapy. All of these persons who failed treatment previously did not have an immunotherapeutic alternative or followup course of inductive treatment which could be expected to be medically effective. The broad aspects and proper usage of the methodology described herein now provides an alternative course of immunotherapeutic treatment for all persons in each of these three categories of failed patients.

II. The Underlying Model Of BCG Action And Cytokine Network Mechanism

Use of the live vaccine strain of Mycobacterium bovis BCG for treatment of superficial bladder cancer is the most successful example of applied immunotherapy against a solid malignancy in this model. Effective intravesical BCG anti-tumor activity relies on two distinct yet interconnected processes: (1) the induction of an appropriate bladder cytokine milieu which phenotypically alters cancer cells to become better immune targets; and (2) the recruitment and activation of effector cells into the bladder to kill these targets. Furthermore, while tumor elimination does NOT appear to involve classic tumor-specific cytotoxic T lymphocytes (CTLs), the mechanism nonetheless is T cell dependent and relies on the induction of a particular cellular immune response known as T helper type one (Th1) in which interferon-gamma (IFN-γ) plays a critical role. The coincident presence of selective

cytokines with BCG in any of its available forms can enhance the recruitment, activation, and perpetuation of this Th1 dependent process leading to higher anti-cancer efficacy.

A new working cytokine network model has been formulated as a result of extensive experimental testing in which additions of stimulatory cytokines or functional subtractions of inhibitory cytokines profoundly synergize the induction of IFN-γ by BCG. This is shown by Fig. 1. In the model the width of the arrows indicates the relative importance of each cytokine contributing to IFN-γ release. A substantial body of data has also been collected from in-vitro and in-vivo experiments in mice implicating a critical role for Th1 responses and, in particular, the induction of IFN-γ as part of BCG anti-tumor activity. These observations have been verified in human peripheral blood mononuclear cell (PBMC) assays and through analysis of clinical urine samples obtained from patients undergoing conventional BCG therapy.

The experimental basis for the derivation of this cytokine network model critical for effective BCG anticancer activity is provided in the following 4 sections.

1) IFN-γ is a highly regulated distal product of multiple interacting proximal cytokines induced by immune cell stimulation with BCG.

BCG was found to induce multiple cytokines in-vitro, in-vivo and clinically including: IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α, GMCSF, IP-10, and IFN-γ. IL-4 was only detectable using highly sensitive RNA PCR methods. Kinetic analysis revealed that these cytokines are produced in a sequential manner. See Figs. 2A and 2B. While there are some clear differences between the murine splenocyte and human PBMC assays, in both cases IFN-γ is among the last cytokines produced. This is consistent with a cascade of proximal cytokines leading to IFN-γ that is indirectly regulated by the timely appearance of down-modulatory IL-10. The dependence of IFN-γ on these proximal cytokines was verified by antibody neutralization in culture (see Figs. 3A and 3B) and the ability to recapitulate progressively higher IFN-γ production by adding purified combinations of proximal cytokines to murine splenocytes that mimic their natural appearance

following BCG therapy (Fig. 4). Adherent cells (primarily macrophages - a known source of IL-12) mixed with $CD4^+$ or $CD8^+$ cells were found to be essential for significant IFN-γ production. A positive feedback loop was identified between IFN-γ and IL-12 further potentiated by IL-18. IL-2 increases during BCG rechallenge as the result of immune memory and serves as a natural accelerator during sequentially administered BCG as it is currently applied therapeutically for bladder cancer.

To test this model during clinical BCG therapy, select urinary cytokines were analyzed for the first 12 hours following each weekly treatment in several patients. The result in one such patient as examplified by Fig. 5. From this analysis it is apparent that IFN-γ, IL-2, and IL-12 (all Th1 cytokines) rise closely in parallel. IL-10 is displaced roughly 4 weeks after the rise in IFN-γ suggesting it has a functional rheostatic role in quenching the IFN-γ cascade.

2) The IFN-γ amplification pathway can be markedly enhanced by the addition of dose-limiting proximal cytokines, certain exogenous cytokines, and antibodies to IL-10.

Investigations using BCG treated immune cell cultures revealed that certain proximal stimulatory cytokines such as IL-6 and TNF-α are normally present in functional excess while others may be functionally dose limiting. In mice, significant enhancement of BCG-induced IFN-γ release was seen with additions of GMCSF, IL-2, IL-12, and/or IL-18. The highest degree of amplification with single cytokines in mice was achieved by IL-18 (350 X) followed by IL-12 (100 X) while IL-2 and GMCSF provoked only 5-10 X increases. Furthermore, half maximal levels of stimulation for IL-18 and IL-12 were achieved with under 10 pg/ml while 20-50 fold higher concentrations of IL-2 or GMCSF were required. Further, 5-10 fold increases of IFN-γ could be achieved when IL-18 and IL-12 (both at 10 pg/ml) were added together. Similar degrees of synergy with IL-2 plus IL-12 could be obtained but required 20-50 fold higher amounts of IL-2.

By contrast, the data revealed the major dose-limiting co-stimulatory cytokines for BCG augmentation in humans are IL-12 and IL-2; supplemental GMCSF was NOT found to be beneficial while the addition of IL-18 provided only

marginal benefit. Further empiric testing shown in Fig. 6 revealed that the addition of exogenous cytokines even while not produced to any measurable extent during BCG therapy could have profound effects on IFN-γ production from BCG stimulated human PBMCs ranging from strongly inhibitory to strongly stimulating. For example, natural and recombinant IFN-α and β were found to be substantially enhancing (likely due to its inhibitory effects on IL-10 production). Furthermore, as shown by Figs. 7A and 7B, substantial (>2-3 X) effects are achieved with the single addition of as little as 4 pg/ml of IL-12, 25 pg/ml (~1 IU/ml) of IFN-α, or 300 pg/ml of IL-2. More importantly, the combination of these very low doses of IL-12 and IFN-α with BCG further doubles or triples the response. Under these circumstances even minute amounts of IL-2 at 10 pg/ml are further co-stimulatory. Finally, with a mere 20 pg/ml of IL-12 plus 250 pg/ml of IFN-α (or plus 300 pg/ml IL-2), an additional 9 fold (10-fold) rise in IFN-γ is achieved, representing a net 60 fold (100 fold) rise from baseline BCG alone. Even more remarkable, under these conditions, a 1000 fold drop in BCG concentration still maintains over 50% of this maximal activity.

The addition of anti-IL-10 antibody to either murine or human immune cell cultures also enhanced IFN-γ as much as the addition of the weakly stimulatory cytokines, reinforcing IL-10's role as a functional down-modulator of BCG induced IFN-γ and suggesting a further means by which the Th1 pathway may be promoted. However, the failure of IL-10 neutralization to recapitulate the extremely high levels of IFN-γ (up to 1000-fold) achieved with the strong stimulators demonstrates the complexity of this synergy mechanism.

In-vivo murine experiments confirmed that intravesical administration of BCG plus IL-12 invokes the same synergy for producing IFN-γ in the mouse urine as seen during in-vitro cultures [O'Donnell et al., J. Immunol. 163: 4246 (1999)]. Similar in-vivo reinforcement was evident in a patient treated with low dose intravesical BCG plus IL-12 where a 50-fold increase in urinary IFN-γ was demonstrated. Subsequently, clinical trials with combined intravesical BCG plus exogenous IFN-α were initiated. At a median follow-up of 21 months, 56% of patients that have failed prior BCG monotherapy are disease-free (a remarkable

achievement considering other salvage therapies are typically <20% effective). These observations show that cytokine-enhanced BCG Th1 stimulatory pathways are directly applicable to clinical practice and can result in major improvements in bladder cancer therapy.

3) Cytokine rBCGs [BCG bacterial cells able to express a recombinant DNA coding for an active form of a specific cytokine sequence] expressing the appropriate murine or human co-stimulatory cytokines show the expected substantial up-regulation of IFN-γ during in-vitro testing.

Cytokine rBCGs expressing murine GMCSF, IL-2, and IFN-γ were each able to substantially augment IFN-γ production while the control non-expressing rBCG (261) and rBCG expressing IL-6 were not significantly different from wild type BCG, as shown by Fig. 8. Importantly, even very small amounts of IFN-γ (~200 pg/ml) secreted by mIFN-γ rBCG activated a positive feedback loop resulting in production of over 10 ng/ml amounts of endogenous IFN-γ.

rBCGs expressing single hIL-2 or hIFN-α have been produced which show ~3 fold greater activity than wt BCG during in-vitro testing. When simply mixed together, they are roughly 10 times as potent. More important, this combination retains substantial potency (6 fold higher than full dose wt BCG; 18 fold higher than an equivalent dose of wt BCG) even when reduced 1000 fold in concentration, as shown by Fig. 9. Furthermore, with this degree of dilution, both IL-2 and IFN-α are calculated to be below 1 pg/ml.

The persistence of synergy at this low free cytokine concentration provides strong support for the existence of paracrine stimulation in which the rBCG cytokine microenvironment stimulates neighboring immune cells. This represents a distinctive advantage of cytokine secreting rBCG over exogenous cytokines delivered with BCG, a situation which may allow very low doses of rBCG to be used with a concomitant increase in relatively safety.

4) IFN-γ is associated with the BCG anti-tumor mechanism.

IFN-γ has a direct and profound effect on murine (MB49 and MBT-2) and human bladder cancer cell lines by suppressing growth; by inducing cytokine expression from the tumor; and by upregulating MHC class I & II antigens, the apoptotic Fas receptor, the ICAM adhesion molecules, and the TNF-α receptor. Neutralizing antibodies to IFN-γ also accelerate the in-vivo growth of the MB49 bladder cancer in mice (see Fig. 10). Furthermore, the clinical evidence provided by Fig. 11 shows that patients expressing high amounts of urinary IFN-γ (Th1 amplitude) or IFN-γ/IL-10 ratios (Th1 polarity) are more likely to be bladder cancer responders (closed symbols) than non-responders (open symbols). Together these observations suggest that IFN-γ is a good marker of effective BCG therapy and a necessary component in the mechanistic process.

III. The Mycobacterium Species

The present therapeutic treatment system utilizes at least one species or strain of Mycobacterium to be delivered on one or multiple treatment occasions as a component part of the prepared combination immunostimulant. The particular species of choice used as a component may be selected from among M. bovis BCG, an attenuated non-pathogenic (for humans) strain of Mycobacterium, and/or a recombinant DNA strain of Mycobacterium. Each of these will be described in detail hereinafter.

Mycobacterium bovis BCG

Owing to their impact as major human pathogens and as a result of their profound immunostimulatory properties, Mycobacteria have long been intensively studied. In the early 1920s, an attenuated Mycobacterium, Mycobacterium (M.) bovis Bacille Calmette-Guerin (M. bovis BCG or BCG) was isolated for use as a vaccine against tuberculosis [Calmette et al., Acad. Natl. Med. (Paris) 91: 787-796 (1924), reviewed in Collins, F.M., Bacterial Vaccines (R. Germanier, ed.), Academic Press, pp. 373-418 (1984)]. Although the efficacy of this vaccine varied considerably in different trials, and the reasons for its variable efficacy have yet to

be resolved, BCG is among the most widely used human vaccines [Luelmo, F., Am. Rev. Respir. Dis. 125: 70-72 (1982); Fine, P.E.M., Reviews of Infectious Diseases II (supp. 2), 5353-5359 (1989)].

Bacille Calmette-Guerin is a non-virulent strain of M. bovis and has been used as a live vaccine for more than 50 years. In the past 35 years, it has been administered to over 2.5 billion people, with remarkably few adverse effects (e.g., estimated mortality of 60/billion). BCG has been found in numerous studies to have protective efficacy against tuberculosis. It is also the species of choice most preferred for use in intravesical immunotherapy of superficial bladder cancers in humans and animals [See for example: Alexandroff et al., Lancet 353: 1689-1694 (1999) and the references cited therein].

Other strains of Mycobacterium

A number of other, non-pathogenic or non-human infecting strains of Mycobacterium exist and are deemed to be suitable for use in this treatment system. Such strains include M. smegmatis, M. phlei, and M. piscium, respectively in that naturally-existing forms; and attenuated live mutant species of these.

Recombinant BCG and other Mycobacteria species

The recent application of molecular biological technology to the study of Mycobacteria has led to the identification of many of the major antigens that are targets of the immune response to infection by Mycobacteria [Kaufman, S.H.E., Immunol. Today 11: 129-136 (1990); Young, R.A., Ann. Rev. Immunol. 8: 401-420 (1990); Young et al., Academic Press Ltd., London, pp. 1-35, 1990; Young et al., Mol. Microbiol. 6: 133-145 (1992)] and to an improved understanding of the molecular mechanisms involved in resistance to anti-Mycobacterial antibiotics [Zhang et al., Nature 358: 591-593 (1992); Telenti et al., Lancet 341: 647-650 (1993)]. The development of tools that permit molecular genetic manipulation of Mycobacteria has also allowed the construction of recombinant BCG vaccine vehicles [Snapper et al., Proc. Natl. Acad. Sci. USA 85: 6987-6991 (1988); Husson et al., J. Bacteriol. 172: 519-524 (1990); Martin et al., Nature 345: 739-743 (1990);

Snapper et al., Mol. Microbiol. 4: 1911-1919 (1990); Aldovini and Young, Nature 351: 479-482 (1991); Jacobs et al., Methods Enzymol. 204: 537-555 (1991); Lee et al., Proc. Natl. Acad. Sci. USA 88: 3111-3115 (1991); Stover et al., Nature 351: 456-460 (1991); Winter et al., Gene 109: 47-54 (1991); Donnelly-Wu et al., Mol. Microbiol. 7: 407-417 (1993)]. Genome mapping and sequencing projects are providing valuable information about the M. tuberculosis and M. leprae genomes that will facilitate further study of the biology of these pathogens [Young and Cole, J. Bacteriol. 175: 1-6 (1993)].

These developments have created a diverse range and variety of recombinant DNA forms and strains of Mycobacteria, particularly those recombinant species able to serve as a substitute for Mycobacterium bovis Bacille Calmette-Guerin (M. bovis BCG). Merely examplifying these recombinant DNA forms of Mycobacteria and the developments as a whole are the following: U.S. Patents No. 5,866,403 describing the production and uses of homologously recombinant slow growing Mycobacteria; No. 5,854,055 describing recombinant Mycobacteria vaccine vehicles capable of expressing a foreign DNA of interest; No. 5,840,855 describing Mycobacterial recombinants and peptides encoded by the genome of Mycobacterium tuberculosis for use as vectors and protein expression; No. 5,830,475 presenting recombinant Mycobacterial vaccines which express a heterologous DNA encoding a protein or polypeptide product such as a cytokine; No. 5,807,723 offering a homologously recombinant slow growing Mycobacteria and methods of manipulating the genomic DNA of slow growing Mycobacterial species; No. 5,504,005 describing a recombinant Mycobacterial vaccine capable of expressing a foreign DNA of interest against which an immune response is desired; No. 5,591,632 presenting a recombinant BCG Mycobacteria expressing heterologous DNA encoding a polypeptide or protein for initiating an immune response; and No. 5,776,465 describing recombinant Mycobacterial vaccines, particularly a recombinant M. bovis BCG species which expresses heterologous DNA. All of these issued U.S. patents and the references cited within each of their individual texts is expressly incorporated by reference herein.

The role of Mycobacterium species in the bladder cancer treatment system

Although one cannot be precise about the sequence of events in the immune-response cascade elicited after intravesical introduction of a Mycobacteria species (such as BCG) into the bladder, the effects are believed to be as follows. Initially, live BCG organisms bind to the urothelium and infects both cancerous and normal cells. There is strong evidence that the binding is mediated by fibronectin after which the BCG undergoes endocytosis. The interaction of BCG with urothelial cells is thought to result in several immunologically important changes including induction of chemokines such as interleukin-8 (which serve to attract leukocytes to a local site), pro-inflammatory cytokines (granulocyte-macrophage colony-stimulating-factor, tumor necrosis factor α, interleukin- 6), and the upregulation of adhesion-molecule expression (intracellular adhesion molecule 1), which promotes effector cell-tumor cell interactions.

As the regimen of BCG (or other Mycobacterial) treatment continues, a marked infiltration of the bladder wall occurs, which is characterized by the presence of T lymphocytes, macrophages, and neutrophils in the urine, as well as further induction on tumor of intercellular adhesion molecule 1, MHC class I and II molecules, and reversion of cytology from positive to negative. It is believed that the leukocyte infiltrate secretes numerous cytokines, including those that are the hallmark of activated T cells and natural-killer cells (such as interleukin 2 and interferon gamma), which are typically observed. Maximum levels of cytokine secretion, cellular influx, intercellular adhesion molecule 1, and MHC expression and clinical response are usually attained by the fifth and sixth instillation.

The BCG-induced/activated T lymphocytes are considered to be the most important elements of the anti tumor response; and both CD4 and CD8 T lymphocytes are essential for the successful treatment of bladder cancer. Also, BCG-activated killer cells are unique in origin and ability to distinguish between normal and tumor cells. Whether BCG-activated killer cells represent a mixture of two different populations (CD8 and CD56) or a single population that coexpresses markers of T cells and natural killer cells remains unknown.

After BCG treatment on multiple occasions has ended, the activity of the immune system will gradually subside. This is reflected by a decline in infiltrating leukocytes, MHC expression on epithelium, and cytokine levels.

IV. The Selectively Chosen Cytokines

The present immunotherapeutic treatment system utilizes at least one preselected active cytokine in combination with the Mycobacterial species collectively as a combination immunostimulatory agent. The chosen active cytokine(s) may be a prepared-in-advance, discrete substance co-administered with the M. species and introduced at the proper anatomic site in the bladder or upper urinary tract. Alternatively, the selected active cytokine is a recombinant cytokine expressed in-situ by an introduced recombinant Mycobacterial species or by a genetically modified host cell expressing in-situ at least one exogenous DNA sequence encoding the cytokine of choice. Any or all of these alternative formats for introducing the selected active cytokine to the tumor in the living patient's body is suitable for use when practicing the present methodology.

Cytokines, by broad definition, are factors (such as a lymphokine or monokine) produced by mononuclear cells that affect other cells. Cytokines, often acting in serial sequence, serve as endogenous signals between cells which, in conjunction with antigens, rapidly amply both the localized and systemic host defenses and defense mechanisms involving macrophages, lymphocytes, and other activated or naturally-occurring cell types. Among the well characterized cytokines produced and detected locally are those listed by Table C previously herein.

A. Selected Cytokines Suitable For Use

The present bladder cancer treatment method and system purposely causes and requires an introduction of at least one cytokine - and preferably as many as ten different families of cytokines - into the patient's bladder or upper urinary tract (ureter and renal pelvis regions) concurrently and in combination with each therapeutic administration of a Mycobacterial species on each treatment occasion. However, it now must be recognized and understood that not all cytokines

conventionally known to exist or able to be produced are suitable for use in the present cancer treatment methodology. To the contrary, there are major differences in the functional properties and clinical value among the conventionally known cytokines -- differences which cause them to be ranked as superior or inferior as well as useful or non-useful within an ordered system of clinical worth. This ranking and assessment of clinical therapeutic usefulness is based in part on the cytokine network model described previously herein; however, the segregation of cytokines provided herein is the result of empirical evaluation of each individual cytokine's ability to produce a synergistic action on the induction of IFN-gamma in combination with BCG (or other Mycobacterial species) stimulation (see Fig. 6).

It is essential to note and appreciate that only certain specific cytokines will potentiate BCG-induced Th1 activity (IFN-gamma induction) when provided in combination with BCG; and only these are suitable. Human PBMCs stimulated with BCG and a few selected single (or multiple) cytokines will raise IFN-gamma production in-vivo. Moreover, because not all naturally induced cytokines resulting from BCG stimulation are dose limiting, there is no logical way to predict in advance which cytokines would augment IFN-gamma release, or remain relatively neutral, or actually inhibit IFN-gamma production. Similarly, the effect of adding different exogenous recombinant cytokines not normally induced in significant quantity by BCG in-vivo could only be determined empirically. From the subsequently described experiments, a list of preferred co-stimulatory cytokines for clinical use with BCG that maximize IFN-gamma and Th1 activity was developed. These findings are summarized by Table 1 below.

Table 1

Ranked Cytokines

Most Preferred / Most Active (combination > single)	Combination: IFN-α/β + IFN-γ + IL-2/15 + IL-12; IFN-α/β + IL-2/15 + IL-12; IL-2/15 + IL-12 IFN-α/β + IL-2/15; IFN-α/β + Il-12 Single: IFN-α, β, γ; IL-2, IL-12, IL-15
Weakly Active	Single: IL-1 α, β; IL-3; IL-18
Inactive / Neutral	TNF-α, β
Weakly Inhibitory	IL-5, IL-8, MCSF, GCSF
Strongly Inhibitory	TGF-β, GMCSF, IL-4, IL-6, IL-10, IL-13

It will be noted and appreciated that any subtype or isoform, natural or recombinant, of these individually identified cytokines is included by the designates given above. Thus for example, the term "interferon-alpha" includes by definition the subtypes interferon-α2b and interferon-α2a, the synthetic interferon α con-1, and leukocyte derived "natural" interferon-α.

The listing of Table 1 provides information not only on the magnitude of stimulation but also on the probability of achieving substantial stimulation when treating heterogenous cancer patients. For instance, in-vitro experiments show the combination of BCG with IFN alpha or beta + IL-2 + IL-12 achieves over 75% maximal stimulation in almost all patient instances whereas BCG, with single IFN-a or -b results in ~80% of patients having at least a 4-fold amplification with ~50% reaching within 75% of maximal stimulation. By contrast, the addition of IL-18 to BCG amplifies IFN-g in less than 1/2 of patients and by less than 3 fold in all. TNF-α is neutral in almost all cases with rare minor improvements of less than 100%. IL-10 is inhibitory in all cases tested, the extent of which, however, depends on the basal amount of IFN-g induced by BCG alone.

For purposes of practicing the present invention, therefore, only the "Most Preferred/Most Active" group of 6 specific cytokines and the "Weakly Active" group of 4 specific cytokines may be used singly or in multiple blends in

conjunction with the Mycobacterial species of choice for immunotherapeutic purposes. The members of the "Inactive/Neutral" rank, the "Weakly Inhibitory" group and the "Strongly Inhibitory" set are individually, cumulatively and collectively to be avoided clinically and are not to be administered to the patient at any time or used on any immunotherapeutic treatment occasion.

B. The Cytokine(s) Of Choice

It is also important to note how to select which single cytokine or blend of multiple cytokines should be employed as part of the combined immunostimulatory agent preparation. In this context, it is valuable to consider the separate categories of "failed cytokine patients" and "failed BCG + a cytokine patients" as illustrative of the selection problem.

Clearly, the failed patients in each of these categories each received a cytokine alone or in combination with BCG as a therapeutic treatment for their superficial bladder cancer; and, equally important, such prior treatment attempts comprising a cytokine all failed to result in a disease-free state for the patient. In such instances, the present methodology provides a clear and unequivocal cytokine selection process, which is: pick and use a different single cytokine or a blended plurality of cytokines from among those in the allowable listing. A second trial or reduction with that particular regimen is generally to be avoided; however, re-administration of that particular cytokine of the prior failed treatment may be given in conjunction with other members of the Most Preferred/Most Active group. Thus, in view of the "Most Preferred/Most Active" group offering a choice of not less than 6 different effective cytokines to the clinician or physician, at least 5 supplemental choices remain and are available for use. For example, if IL-2 were previously administered to the patient in the prior failed treatment - then any of the 5 other cytokines in this grouping (IFN-α, β, γ, IL-15 or IL-12) may be chosen as used alone or with the others in blends of 2-6 cytokines in admixture.

The selection criterion for a cytokine of choice is similar regarding the four cytokine members constituting the "Weakly Active" grouping. Should the cytokine used previously for the patient in the prior failed treatment attempt be one of these,

either of the two other members of this grouping may be employed presently, alone or as a blending of the others. Also, the entire membership constituting the "Most Preferred/Most Active" grouping also remains available for use, singly or as a blend of up to six different individual cytokines.

C. Formats For The Selected Cytokine(s) Of Choice

Two different broad formats are conventionally known and available for using the selected cytokine(s) of choice. These are: a prepared-in-advance format where the cytokine exists as a discrete substance and composition of matter; and the recombinant cytokines expressed in-situ by a host cell carrying an exogenous DNA segment. In the first prepared-in-advance format, it will be recognized that all of the cytokines of choice individually are well known and characterized in physical structure and properties, formulation and chemical composition and biochemical/ immunological functions and actions. A summary of such properties and characteristics has been provided previously herein by Table C. The scientific and patent literature published over the last 40 years also provides ample information, descriptive detail, and methods for cytokine synthesis and/or isolation and purification for each selected IL-1, IL-2, IL-3, IL-12, IL-15, IL-18, IFN-α, IFN-β, and IFN-γ. These printed publications are examplified and represented by the following, each of which is expressly incorporated by reference herein: Belardelli et al., APMIS 105: 161-179 (1995); Sasaki et al., Blood 85: 1220-1228 (1995); Fan et al., Biochem Biophys. Res. Comm. 225: 1063-1067 (1996); Pestka et al., Ann. Rev. Biochem. 56: 727-777 (1987); Orange et al., J. Immunol. 152: 1253-1264 (1994); Kobayashi et al., J. Exp. Med. 107: 827-845 (1989); Goeddel et al., Nature 290: 20-26 (1981); Familletti et al., Agents & Chemother. 20: 5-9 (1981); Tripp et al., Proc. Natl. Acad. Sci. USA 90: 3725-3729 (1993), see also U.S. Patent Nos. 5,928,636; 5,457,038; 5,648,072; 5,891,432; 5,616,477; 5,601,815; 5,965,379; 5,696,079; and 5,833,976.

In addition, see the following: Geha et al., Cell Immunol. 10: 86 (1974); Morgan et al., Science 193: 1107 (1976); Smith et al., J. Exp. Med. 151: 1551 (1980); Robb et al., Proc. Natl. Acad. Sci. USA 80: 5990 (1993); U.S. Patent Nos.

4,401,256 and 4,473,642; Tamiguelu et al., Nature 302: 305 (1983); Mita et al., Biochem. Biophys. Res. Comm. 117: 114 (1983); Altman et al., Proc. Natl. Acad. Sci. USA 81: 2176 (1984); Rosenberg et al., Science 223: 1412 (1984); Tiggs et al., Science 243: 781 (1989); Gillis et al., Contemporary Topics In Molecular Immunology, vol. 10, "The Interleukins", Plenum Press, N.Y., 1985.

Finally, note the following: Zoon, K.C., Interferon α: 1-12 (1987); Weissman et al., Prog. Nucl. Acid Res. Mol. Biol. 33: 251-300 (1986); Stiem et al., Ann. Inter. Med. 96: 80-93 (1982); Mannering et al., Ann. Rev. Pharmacol. Toxicol. 96: 80-93 (1982).

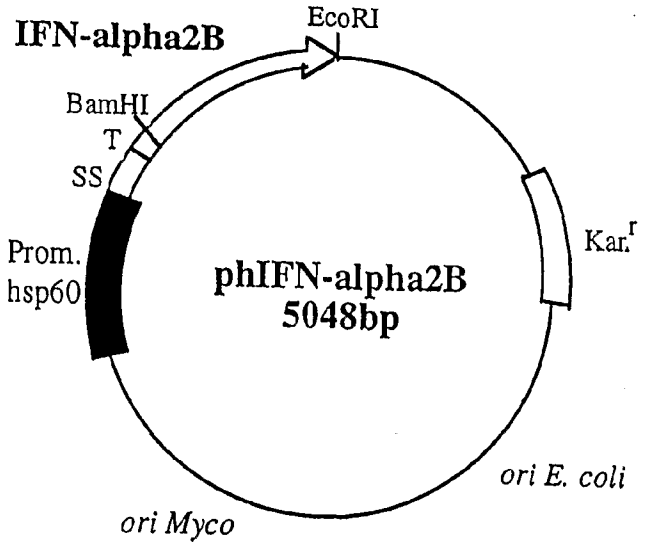
The other broad mode of use is the recombinant cytokine(s) format, as conventionally known and prepared. Typically in this recombinant cytokine format, a host cell has been genetically modified to carry and express in-situ at least one exogenous DNA sequence encoding a single cytokine or a series of different cytokines. The technology for recombinant formats is well documented and described both in the scientific literature as well as in mature patents. In particular, various recombinant DNA Mycobacterial strains have been developed to include and express heterogenous DNA sequences coding for one or more specific cytokines. Describing such recombinant Mycobacterial species able to express such cytokines in-situ and in-vivo are the following, each of which is expressly incorporated by reference herein: U.S. Patent Nos. 5,776,465; 5,591,632; 5,866,403; 5,854,055; 5,840,855; 5,830,475; 5,504,005; 5,807,723; and the references cited within each of these individually.

As a point of information also, it will be recognized and appreciated that in terms of preparing and using recombinant forms of Mycobacterium, it is important, if not essential, that the user be at least familiar with the many established procedures and conventionally known techniques for manipulating and modifying nucleotides and DNA (and RNA) fragments as well as the vectors to carry them which have been reported and are today widespread in use and application. Merely exemplifying the many authoritative texts and published articles presently available in the literature regarding genes, DNA nucleotide manipulation, vectors, and the expression of cytokines from manipulated DNA fragments are the following: Gene

Probes for Bacteria (Macario and De Marcario, editors), Academic Press Inc., 1990; Genetic Analysis, Principles Scope and Objectives by John R.S. Ficham, Blackwell Science Ltd., 1994; Recombinant DNA Methodology II (Ray Wu, editor), Academic Press, 1995; Molecular Cloning, A Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor Laboratory, 1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios Scientific Publishers, 1994; and the many references individually cited within each of these publications. All of these published texts are expressly incorporated by reference herein.

Finally, as merely one preferred example of a recombinant format, a recombinant human IFN-α expression vector is provided below. This prepared expression vector is a plasmid which is used to transform the desired Mycobacterial strain; and will serve for expression and secretion in-situ of a recombinant human form of IFN-α wherever and whenever the recombinant Mycobacterial strain is introduced at a chosen anatomic site in the human body.

RECOMBINANT HUMAN IFN-α EXPRESSION VECTOR



SS: BCG alpha-antigen signal sequence
T: influenza virus hemagglutinin epitope tag sequence

V. The Complete Immunotherapeutic Formulation

The complete formulation of the combination immunostimulatory preparation is at a minimum: a first format comprising a non-pathogenic strain of Mycobacterium (such as M. bovis BCG) in admixture with at least one cytokine selected from the group consisting of IL-1, IL-2, IL-3, IL-12, IL-15, IL-18, IFN-α, IFN-β, and IFN-γ; and a second format comprising a recombinant Mycobacterium species carrying and expressing in-situ at least one exogenous DNA sequence (in integrated or non-integrated form) encoding not less than one cytokine selected from the same selective grouping. Mixtures of recombinant Mycobacterium species are likewise permitted.

In the first format type, it is expected that the live Mycobacterial cells will be combined with a synthesized and/or purified cytokine(s) of choice in quantitative ratios as described hereinafter by the illustrative treatment protocols. Thus, the viable Mycobacterium cells and the cytokine(s) of choice may be given concurrently, but as individual preparations; but preferably are combined in admixture and co-administered as a blended mixture to the prechosen anatomic site in the patient's urinary system as a therapeutic agent.

In the alternative format instance, the recombinant Mycobacterium strain (having been genetically modified in advance to carry and include a DNA sequence encoding at least one preselected cytokine) is itself the cellular vehicle and the prepared formulation for immunotherapeutic treatment of the patient. Upon introduction of the viable recombinant Mycobacterial cells to the bladder or the upper urinary tract of the patient, the live Mycobacteria will express and secrete the selected cytokine(s) of choice in-situ at the anatomic site as an active substance. In this manner, by causing the introduction of the living recombinant Mycobacteria, a concomitant in-situ introduction of the chosen cytokine is caused which is concurrent with the administration of the live Mycobacteria. Thus the introduction of recombinant cells also intrinsically causes and achieves a concurrent introduction of the selected cytokine in due course of time.

Routings And Manner Of Administration

Administration of the immunotherapeutic agent(s) into the bladder is generally accomplished by the atraumatic insertion of a catheter into the bladder. The liquid therapeutic agent(s) usually in a volume between 25-100 ccs are then delivered through the catheter into the bladder under low pressure either by gravity or gentle pressure upon which the catheter is generally immediately removed allowing the liquid medication to remain inside. The immunotherapeutic is then retained for 1-3 hours before evacuation by physiologic voiding or catheter reinsertion if voiding is impaired. During induction treatment, sequential weekly or biweekly administration is given totalling 5-12 (but most usually 6) separate administrations. Maintenance treatment may be given according to various schedules but most often as a 3-dose weekly miniseries at intervals of 3-6 months or as a once monthly administration.

Administration of the immunotherapeutic agent(s) into the upper tract may be accomplished via either an antegrade or retrograde fashion. In the former case, it is delivered via a percutaneous tube pre-inserted into the renal pelvis. In the latter case, a stent may be placed in a temporary or more durable manner to provide access from below into the renal pelvis. The agent(s) are then administered under low pressure, such as by gravity drip, at a slow continuous rate, usually over 1-4 hours. The frequency of sequential administration for induction or maintenance treatment is similar to that described above for intravesical administration.

VI. Illustrative Treatment Protocols

Merely to examplify and illustrate the mode and manner of clinical usage for the present immunotherapeutic treatment method, a representative scheme of usage directed to three separate categories and sub-groups of human patients is presented. For descriptive purposes only, Group I is constituted of superficial bladder cancer patients who, individually and collectively, are immuno-pristine and have not previously received any immunostimulatory treatment for their cancer, particularly no induction of BCG or any other Mycobacterial species.

In comparison, Group II is constituted of superficial bladder cancer patients, each of whom has previously undergone at least one immunostimulatory treatment with BCG on a prior occasion, but has failed to respond adequately to such treatment. These patients comprise the category of "failed BCG patients" as defined herein.

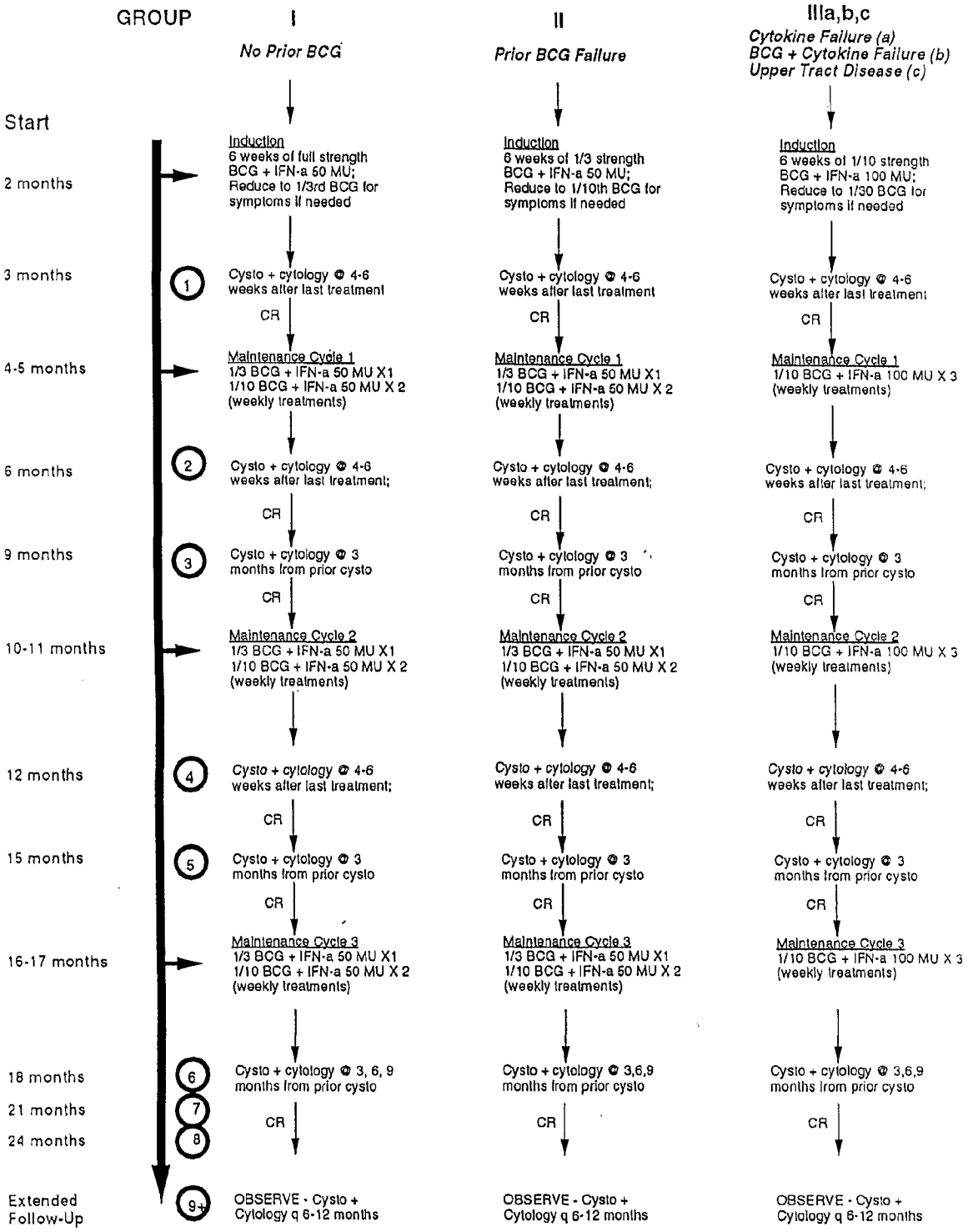
Finally, Group III is constituted of three different subgroupings of human patients: Group IIIa comprises patients who have previously received cytokine monotherapy as a stimulatory agent for treatment of superficial bladder cancer, but have failed to achieve a disease-free state of any duration. This Group IIIa thus forms the "failed cytokine patients" category as described and defined herein. Group IIIb is constituted of patients afflicted with superficial bladder cancer, each of whom has previously received BCG + a cytokine as a therapeutic treatment on at least one prior occasion - but each of which has failed to achieve a disease-free state. This Group IIIb collectively includes the "failed BCG + cytokine patient" category as defined and described herein.

Lastly, Group IIIc is constituted of patients suffering from cancer of the upper urinary tract, whether or not they have received prior BCG or prior cytokine monotherapy before. It should be appreciated that immunotherapy has hitherto not been conventionally used in this patient category.

A. Scheme For Treatment

A general scheme and immunotherapeutic treatment protocol for the present methodology is presented by the schema given below. The immunostimulatory agent employed in these illustrative protocol examples is BCG+IFN-α in each instance.

Schema for BCG + IFN-α Use



Schema:

Induction treatments shown by Table 3 consist of one of three representative regimens based on the patient's prior BCG exposure, his BCG tolerance, and his tumor location. Patients that have not been treated with BCG before (Group I = No Prior BCG) receive standard full-dose BCG plus 50 MU of recombinant IFN-a-2B weekly for 6 treatments. Those that have failed BCG before but did not have to discontinue due to intolerance (Group II = Prior BCG Failure, Non-Intolerant) are treated with one third dose of standard BCG mixed with IFN-a-2B (50 MU) administered weekly for 6 weeks. Finally, prior cytokine failure patients (Group IIIa) are treated with 6 weeks of 1/10th standard dose BCG plus 100 MU of IFN-a-2B. Patients failing a prior induction cycle of combination BCG plus a cytokine (Group IIIb) may receive a 2nd induction cycle if clinically appropriate. Patients with upper tract transitional cell carcinoma regardless of prior therapy would also be treated with the same regimen (Group IIIc). If treatment intolerance occurs in any group during the induction period, the patient may optionally be given a 2-week rest followed by re-initiation of treatments at a BCG dose of roughly 1/3 that of the prior dose. Similar 2-week delays are permitted for repeat episodes of intolerance. Further, BCG dose reduction by 1/3 intervals are used as necessary.

Intravesical therapy is delivered weekly via a temporarily placed foley catheter for a total of 6 induction treatments for patients with bladder TCC. For those with upper tract TCC, a small (usually 4 French) temporary external stent is placed cystoscopically from the bladder into the mid renal pelvis when possible. Alternatively, treatment may be given through a percutaneous nephrostomy tube or other similar device to deliver material into the renal pelvis. All patients are asked to reduce fluid consumption the night and morning before the treatment to facilitate holding the 50 cc of liquid medication for approximately 2 hours and/or minimizing excess dilution. They then void into the toilet. Excessive fluid consumption for the day of the treatment is discouraged.

Standard cystoscopic and cytological evaluations are performed at roughly 3-month intervals during the first 2 years although 6-month intervals may be appropriate during the second year for patients with less aggressive disease.

Progression at any time to muscle invasive disease or metastatic disease require this treatment to be terminated. Recurrence of disease which is surgically resectable and/or intravesical therapy amenable after the first induction course may be offered a second course of induction therapy beginning with an even lower dose of BCG (1/10th standard dose) and a higher dose of IFN-a (100 MU) or an alternate BCG plus cytokine regimen. However, the decision to re-treat is left to the clinician's judgment. In general, patients with non-aggressive bladder tumor (mucosal, low-intermediate grade) will be more suited for re-treatment while only the exceptional patient with recurrent aggressive disease (Stage T1, grade 3, or CIS) should be considered.

After an evaluatory cystoscopy (with a biopsy if indicated) roughly 4-6 weeks after the last induction treatment, patients with no evidence of cancer are given their first maintenance cycle of 3 weekly treatments at the doses indicated in the Schema (roughly 4-5 months after the first induction dose). As long as a complete response is maintained, two additional maintenance cycles are given roughly 6 months apart at approximately 10-11 and 16-17 months post the start of intravesical therapy. Failure to complete any or all maintenance treatments due to treatment intolerance is permitted. Patients that have relapsed with recurrent bladder cancer after initiating maintenance therapy may be offered a different cytokine augmented regimen (such as reduced BCG plus IL-12 if appropriate).

B. Patient Selection Criteria

Patient Characteristics:

In the United States most patients with bladder cancer are over 60 years of age, and many are smokers. The age-adjusted incidence of bladder cancer is approximately 17 per 100,000. Bladder cancer is 3.7 times more common among men than women and approximately 2-fold higher in white men than black men and 3-fold higher in white men than Hispanic men. The incidence of upper tract TCC is roughly 4% that of bladder cancer but is 3-5 times higher in patients with advanced bladder cancer, those with long duration disease, or patients successfully treated with BCG for aggressive superficial bladder cancer.

Approximately 70-80% of cases present initially as superficial disease (Stage Ta, T1, and CIS). Roughly 25-30% of superficial cases are of the aggressive subtype (Stage T1, grade 3, CIS). Patients may be of either sex and any race and have superficial TCC only. In addition, while potentially all patients with superficial TCC of the bladder are eligible for this treatment protocol, given the vacuum of acceptable and efficacious treatments for BCG failure patients and those with upper tract disease, it is likely that these persons will be over-represented.

Pre-Exclusion Criteria:

1) Females who are pregnant or breast feeding.
2) Patients who presently have muscle invasive bladder cancer, disease within the prostatic stroma, or prior evidence of metastatic TCC would not be appropriate for this treatment. Previous definitive resection by partial cystectomy, nephroureterectomy, or prostatectomy does not exclude patients from consideration as long as current disease is superficial.
3) Active treatment with any cytotoxic, immunosuppressive or chemotherapeutic agent.
4) Papillary or solid TCC resection, bladder biopsy, or TURP within the previous 7 days or evidence of gross hematuria within the previous 2 days to minimize the chance of inadvertent BCG intravasation into the bloodstream.
5) Serious concurrent infection and particularly no evidence of active tuberculosis.
6) Any significant medical or psychiatric illness that would prevent the subject from complying with the protocol.
7) Unstable cardiac disease including angina, ischemia, arrhythmia, and/or congestive heart failure that might be precipitated by occasional fevers and chills caused by the treatment.

Post-Exclusion Criteria:

After onset of therapy, a patient should be excluded from further treatment for any of the following reasons:

1) Progressive disease to stage T2 or higher at any evaluation point.

2) Need for pelvic radiation or treatment with any immunosuppressive or cytotoxic chemotherapeutic agent for other malignant or non-malignant conditions.

3) Females who become pregnant.

4) Serious infection, or major surgery or any other reason that interferes with treatment.

5) Documented serious and recurrent adverse reaction to the administered agents.

6) Subjective toxicity or unrelated personal reasons.

C. Potential Risks

BCG - Intrinsic Risks:

Bacillus Calmette-Guerin (BCG) is widely regarded as the most effective intravesical agent to prophylax against recurrent superficial bladder cancer, treat residual papillary tumor, and treat carcinoma in-situ (CIS). Mild-moderate toxicity is experienced by many patients consisting of irritative bladder symptoms (frequent and painful urination lasting less than 3 days), blood in the urine, fever, fatigue, or flu-like symptoms lasting less than 3 days. Serious but infrequent (<5% incidence) reactions include the following: skin rash, arthritis, urinary tract infection, shrinkage of the bladder, obstruction of the kidney urinary tube (ureter), inflammation of the prostate gland and testicles, inflammation of the liver, and kidney abscess. On rare occasions (approximately 1 in 250 patients), BCG can cause severe systemic infection. Such an occurrence may require hospital admission, intravenous antibiotics and up to 3-6 months of oral anti-tuberculosis medication. In extreme cases such severe infections could result in breathing difficulties, shock, or even death.

Interferon-alpha - Intrinsic Risks:

Interferon-alpha (IFN-a) by itself when instilled directly into the bladder has generally been very well tolerated. Occasionally mild cystitis, chills, and transient

fever or flu-like symptoms may occur in the minority (<20%) of patients. Dose-limiting toxicity is not reached even at the highest dose of 1000 MU/week, well below the 50-100 MU/week dose in this clinical protocol.

Cancer Risk from Proposed Intravesical Therapy Format:

The risk of cancer progression must be carefully considered and discussed during the informed consent process, especially for those patients that have failed previous BCG therapy. For patients with non-aggressive superficial disease of the stage Ta, grade 1-2 subtype, the risk of progression to invasive disease during the induction timecourse is very small (<5%). For those patients with aggressive histology, however, there is a chance of interval muscle invasion that may approach 30% in some cases and a finite chance of metastasis perhaps up to 5%. The decision to opt for this treatment versus a higher morbidity therapy involving radical surgery, high dose systemic chemotherapy, and/or radical radiation therapy must take into account age, co-morbidity, and patient desires.

Reduction of BCG dose to 1/3 for induction treatments as proposed is supported by the clinical response equivalence between 1/3 and full dose BCG. Further, BCG dose reduction for treatment intolerance is justified by data showing the maintenance of high urinary IFN-g levels when IFN-a is administered with BCG plus the known activity of IFN-a alone. The use of maintenance therapy for responders is supported by the results of the SWOG 8507 trial [Lamm, D.L., Eur. Urol. 27 (suppl. 1): 2 (1995)]. BCG dose reduction allows more patients to tolerate this therapy. Finally, because maintenance therapy is still controversial, variably practiced, and adds to local toxicity, it should not be an absolute requirement.

D. Procedures To Minimize Patient Risks

Minimizing Risk of Serious BCG Infection:

As most cases of serious BCG infection are precipitated by traumatic intravesical catheter insertion, only experienced medical personnel should perform drug instillation. The drugs should be delivered by gravity instillation to prevent high pressure entry into the bloodstream. For moderately severe symptoms (usually

grade 3 toxicity), BCG dose reduction and/or treatment delay may be employed. For symptoms suggestive of serious BCG infection such as fever over 103 F with shaking chills, patients are instructed to return to their medical center for evaluation. Any patient suspected of BCG sepsis is admitted to the hospital for monitoring and initiation of appropriate supportive and specific anti-Mycobacterial drug therapy. Further BCG treatment will be withdrawn.

Minimizing Risk of Cancer Progression:

Patients are monitored for bladder cancer recurrence/clinical response based on an every 3 month visual cystoscopic and cytological evaluation during the first 2 years of treatment as is the standard-of-care in most communities. Upper tract disease is monitored by dye studies and cytologic washings looking for malignant cells. Any progression to invasive disease requires immediate withdrawal from further treatment in favor of other alternatives (often cystectomy or nephroureterectomy).

VII. Experiments And Empirical Data

The following is a series of summations presenting the sum and substance of many different in-vitro and in-vivo experiments as well as a range of different clinical human case studies. For ease of understanding and presentation clarity of information purposes, a minimal recitation of the typical methods and materials background is given. It will be recognized that the various individual in-vitro test and assay techniques and protocols are conventionally described in the scientific and medical/clinical published literature. The human clinical studies are recited in substantive detail.

Attention is directed also to several 1999 published papers which describe some preliminary in-vitro experiments and test protocols. These publications are: O'Donnell et al., J. Immunol. 162: 2399-2405 (Feb., 1999); O'Donnell et al., J. Urol.: 286, Abstract 1108 (April, 1999), Alexandroff et al., Lancet 353: 1689-1694 (May, 1999); O'Donnell et al., J. Immunother. 22(5): 463, Abstracts (Nov. 1999);

O'Donnell et al., J. Immunol. 163: 4246-4252 (Nov., 1999). All of these publications, individually and cumulatively, are explicitly incorporated by reference herein.

Materials and Methods:

BCG

MV261 BCG, a Pasteur strain previously transfected with the kanamycin resistance plasmid pMV261 (36) was used in the in-vitro experiments. This strain had been shown to possess very similar immunostimulatory properties to that of commercial lyophilized BCG preparations. This BCG strain was routinely kept at 37°C in 7H9 Middlebrook broth (Difco, Detroit, MI) supplemented with 10% albumin dextrose concentrate (5% BSA, 2% dextrose and 0.85% NaCl), 0.05% Tween 80 (Sigma, St. Louis, MO), and 30 μg of kanamycin/ml. One unit of absorbance at 600 nm for the BCG culture was calculated as 2.5 X 10^7 CFU. For clinical intravesical therapy, lyophilized preparations of BCG (TheraCys; Connaught Pasteur Merieux, Ontario, Canada) was used.

PBMC culture

In accordance with approved clinical protocol, blood samples were collected from bladder cancer patients before intravesical BCG therapy and immediately before the 6th intravesical dose. PBMCs were prepared from buffy coat leukocytes purified on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Viability by trypan blue exclusion usually exceeded 95%. PBMCs were suspended in RPMI 1640 medium containing 10% FCS and 30 μg/ml of kanamycin, and incubated at 37°C in a humidified 5% CO_2 incubator at a density of 8 X 10^5 cells/200 μl/well in 96-well tissue culture plates in the presence or absence of designated doses of BCG, IFN-α2B, or both. In most experiments, an IFN-α2B concentration of 1 X 10^5 IU/ml was selected to approximate the concentration applied clinically into the bladder. In some experiments human recombinant cytokines IL-12 (Genetics Institute, Cambridge, MA) and IL-10 (PharMingen, San Diego, CA) or neutralizing Abs: goat anti-human IL-12 (R&D Systems, Minneapolis, MN), mouse anti-human IL-10

(R&D Systems), and rabbit anti-human IFN-α (Pepro Tech, Rocky Hill, NJ) were further used to determine the immune pathway of IFN-α action. The plates were incubated for 72 h and then frozen at -70°C until cytokine ELISA assays were performed.

Urine samples

Previously, the assay of voided urine samples collected from different times after intravesical BCG therapy for IFN-γ revealed that >90% of urinary IFN-γ appeared within the first 2-12 h after therapy. Thus, voided urine during that period of time was collected and pooled for later analysis. Urine samples were stabilized during patient collection with a concentrated buffer containing 2 M Tris-HCl (pH 7.6), 5% BSA, 0.1% sodium azide, and four protease inhibitors (aprotinin, pepstatin and leupeptin at 0.01 mg/ml for each and 4-(2-amino ethyl) benzenesulfonyl fluoride (AEBSF) at 0.1 mg/ml; all purchased from Sigma). At the end of collection, the volume of the 10-h urine was recorded. A 10-ml sample was further preserved by the addition of a protease inhibitor mixture tablet (Boehringer-Mannheim, Mannheim, Germany) and then stored at -70°C before batch analysis for cytokines by ELISA.

ELISA assays and reagents

ELISA reagents including recombinant human cytokines and paired monoclonal capture and detecting Abs for the cytokines were obtained from Endogen (Cambridge, MA) for IFN-γ, from Genetics Institute for IL-12, and from PharMingen for TNF-α, IL-6, and IL-10. Samples of conditioned PBMC cultures and urine collections were assayed by ELISA using a sandwich format according to the manufacturer's instructions. Cytokine concentrations were calculated in standard mass/volume format using standard curves derived from purified recombinant cytokine standards. For all of the above measured cytokines, one IU is equal to ~50-100 pg of purified cytokine.

Cytokines

Highly purified, endotoxin-free, recombinant murine and human IL-12 were provided by Genetics Institute (Cambridge, MA). Goat polyclonal neutralizing anti-murine IL-12 p70, rabbit anti-human IL-12 p40, and monoclonal anti-human IL-12 p40 (clone C11.5.14) Abs were also obtained from Genetics Institute. Recombinant murine IL-10 and neutralizing anti-murine IL-2 were purchased from PharMingen (San Diego, CA). Paired monoclonal ELISA capture and detecting Abs for murine (IL-2, IL-4, and IL-10) and human (IL-4 and IL-10) were also obtained from PharMingen. Paired human and murine IFN-γ Abs and a human IL-2 ELISA kit were obtained from Endogen (Boston, MA). Hayashibara (Okayama, Japan) supplied polyclonal neutralizing Ab to murine IL-18, while polyclonal neutralizing Ab to human IL-18 was purchased from R&D Systems (Minneapolis, MN).

Mice

Female C57BL/6J mice were obtained at 6-8 weeks of age from The Jackson Laboratory (Bar Harbor, ME) and were housed at 20°C with a 12-h light cycle in the Animal Research Facility at Beth Israel Deaconess Medical Center. Mice were acclimated for at least 1 wk before use. Animal care was provided in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, 1985).

In-vitro murine splenocyte and human PBMC cultures

Murine spleens were removed under sterile conditions, minced, filtered through a fine nylon mesh, and placed in ACK lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1mM Na_2EDTA, pH 7.4) to remove RBC. Pooled cells from usually three to five animals were then resuspended in complete RPMI 1640 medium containing kanamycin (30 μg/ml) and transferred at a final concentration of 1-4 X 10^6 cells/ml, depending on the experiment, to 24- or 96-well tissue culture plates containing the appropriate stimulus to be tested. Viability by trypan blue exclusion always exceeded 90%. Human PBMCs were prepared from buffy coat leukocytes purified on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and cultured

under similar conditions as mouse splenocyte cultures. Supernatants were harvested after 72 h of stimulation unless otherwise stated and were frozen at -70°C before batch testing in cytokine ELISAs.

Intravesical drug administration and urine recovery in mice

Mice were anesthetized by the i.p. administration of ketamine/xylazine/ acepromazine stock solution at a dose of 0.2 ml/10 g of body weight. The stock solution was prepared by combining 1.5 ml of ketamine with 0.75 ml of xylazine and 0.5 ml of acepromazine (all products from J.A. Webster), and the resulting 2.75-ml volume was then mixed with 35.75 ml of sterile water and kept at room temperature before use. Under these conditions mice remain asleep for ~1-2 h.

Bladders were catheterized with a 24-gauge Teflon i.v. cannula lubricated in glycerol. After aspiration of all remaining urine, 0.1 ml of drug was administered. Drug was retained by maintaining the cannula within the bladder with a 1-ml Tb syringe attached. After 1 h the cannula was removed, and mice were allowed to void normally. Mice were placed in metabolic cages overnight (15 h) with ample water but no solid food. Urine was collected in a recovery tube on ice containing 0.1 ml/mouse of a 10X urine stabilizer solution (2 M Tris-HCl (pH 7.6), 5% BSA, 0.1% sodium azide, plus 1/2 COMPLETE protease inhibitor tablet (Boehringer-Mannheim, Germany)). Mice produced an average of 0.5-1.0 ml of urine/mouse during this time. After collection the urine was spun to remove any solid debris and was stored at -70°C until batch ELISA cytokine measurements were performed.

Experimental Series I

Cytokine supplementation experiments have identified several cytokines that are profoundly synergistic with BCG in inducing IFN-γ in-vitro, in-vivo, and clinically.

a) In the murine in-vitro system, GMCSF, IL-2, IL-12, and IL-18 (formerly IGIF: interferon gamma inducing factor) are all able to induce high levels of IFN-γ (3-50-fold increase) when added to immune cell cultures simultaneously with BCG. This data is shown by Fig. 12. Substantially higher amounts are induced if combinations of agents are included. By contrast, although TNF-α is important in inducing IFN-γ responses to BCG, additional TNF-α does not raise IFN-γ levels suggesting it is already induced in saturating amounts (data not shown).

b) In the human in-vitro PBMC system, IFN-α, IFN-β, IFN-γ, IL-2, IL-12 and IL-15 are also profoundly co-stimulatory alone and in combination. This is shown by the data of Figs. 6, 13A and 13B. IFN-α works in part by reducing IL-10 production and increasing IL-12 production leading to a net change in favor of Th1 cytokine polarity. By contrast, murine IFN-α is a Th2 polarizer in the murine system (Figs. 14A and 14B) and again highlights important differences between man and mouse and the absolute need to do parallel studies.

The single most active human cytokine in synergy with BCG appears to be IL-12, however, the extent of synergy varies greatly depending on the level of pre-existent sensitivity to BCG. Thus, those with low initial responses (Fig. 13A - PPD negative) may have massive responses (500X) while those already close to maximum stimulation may only achieve a modest percentile boost (Fig. 13B). In this way co-stimulatory cytokines "level the playing field" by bringing up inadequate Th1 responses to BCG alone.

c) In-vivo murine experiments where BCG plus IL-12 are directly placed into the bladder confirm in-vivo synergy for producing IFN-γ in the mouse urine (Fig. 15). Co-administration of BCG plus IL-12 also resulted in a decreased bladder tumor load in a subcutaneous murine bladder cancer model (Fig. 16).

d) In a pilot clinical trial of BCG plus exogenous IFN-α, one can detect a shift toward Th1 polarity (IFN-γ/IL-10 ratio) in the blood and urine of most human patients compared to BCG alone. This combination treatment itself achieved an unprecedented 58% complete response rate at 2 years for patients with superficial bladder cancer that have failed previous BCG therapy. However, for patients that fail this combination, preliminary experiment evidence shows that other cytokines such as IL-12 may be useful.

e) One such human patient who failed BCG plus IFN-α was treated on a compassionate use basis with intravesical IL-12 plus BCG. His in-vitro PBMC testing revealed a 600-fold increase with combination IL-12 plus BCG versus BCG alone that was increased to 1000 fold by the addition of IL-2 to the mixture. This is shown by Fig. 17A. Furthermore, as proof of principle this patient showed a profound 50X increase in his urinary IFN-γ during therapy compared to the results previously obtained with BCG plus IFN-α. This data is shown by Fig. 17B.

f) rBCG expressing the appropriate murine or human co-stimulatory cytokines show the expected substantial up-regulation of IFN-γ during in-vitro testing. This is evidenced by the data of Fig. 8. rBCGs expressing murine GMCSF, IL-2, and IFN-γ were able to substantially augment IFN-γ production while the control non-expressing rBCG (261) and rBCG expressing IL-6 were not significantly different from wild type BCG.

Likewise, rBCG expressing either human IL-2 or IFN-α is a more potent stimulator of IFN-γ from human PBMCs. This data is shown graphically by Fig. 18.

Experimental Series 2:

The Ability Of Cytokine rBCG To Enhance The Immunotherapy

Of Experimental Bladder Cancer In Animal Models

The ability to adequately provide proof of principle that appropriate cytokine rBCG will be better agents for bladder cancer immunotherapy has been hampered somewhat by lack of the most active cytokine rBCGs (e.g., IL-12 rBCG and IL-18 rBCG). However, using exogenous IL-12, it is demonstrated that IL-12 plus BCG is synergistic in the murine bladder tumor model (Figs. 15 and 16). Furthermore, IP-10 secreting cytokine rBCG (IP-10 is both a T cell chemokine as well as an anti-angiogenic cytokine that is induced by IFN-γ) does result in tumor growth delay in-vitro (Fig. 19). The experiments also confirm the general principle that cytokines synergistic for IFN-γ are superadditive in anti-tumor immunity. Multiple mixtures of cytokine rBCGs provide the greatest in-vivo activity. These cumulative results reveal that the best anti-tumor results may require multiple expression of synergistic cytokines from rBCG.

Experimental Series 3:

Untreated Human Patient And Failed BCG Patient Clinical Studies

This human clinical trial included naive, untreated human patients and failed BCG patients to evaluate the immunotherapeutic efficacy and value of BCG + IFN-α as a treatment regimen. One category tested were naive, human patients suffering from superficial bladder or upper urinary tract cancers. The second category were failed BCG patients - those persons who previously received BCG alone as an immunostimulatory therapy on one or more occasions previously, but failed to achieve a disease-free state of any meaningful duration. The general patient characteristics of these persons is given by Table E1 below.

Table E2

Open Label Study of BCG + IFN-α:
Patient Profile

(i) No. Treated = 68; No. Evaluable = 60

(ii) F/u = 21 m (mean); 20 m (median); 8-47 m (range)

(iii) High Risk Population (98% 1 or more risk factors)

- ♦ 93% multifocal (> 2x)
- ♦ 78% dz. present at Rx start (CIS - 67% or pTCC - 12%)
- ♦ 75% aggressive histology (CIS, T1, Gr 3)
- ♦ 63% prior BCG failures (1-3x)
- ♦ 55% multiple recurrent (> 2x)
- ♦ 27% long duration of Bl Ca (> 4 yrs)

The clinical treatment protocol in summary and detailed format using BCG + IFN-α is given below.

Table E2

BCG + IFN-α Treatment Plan

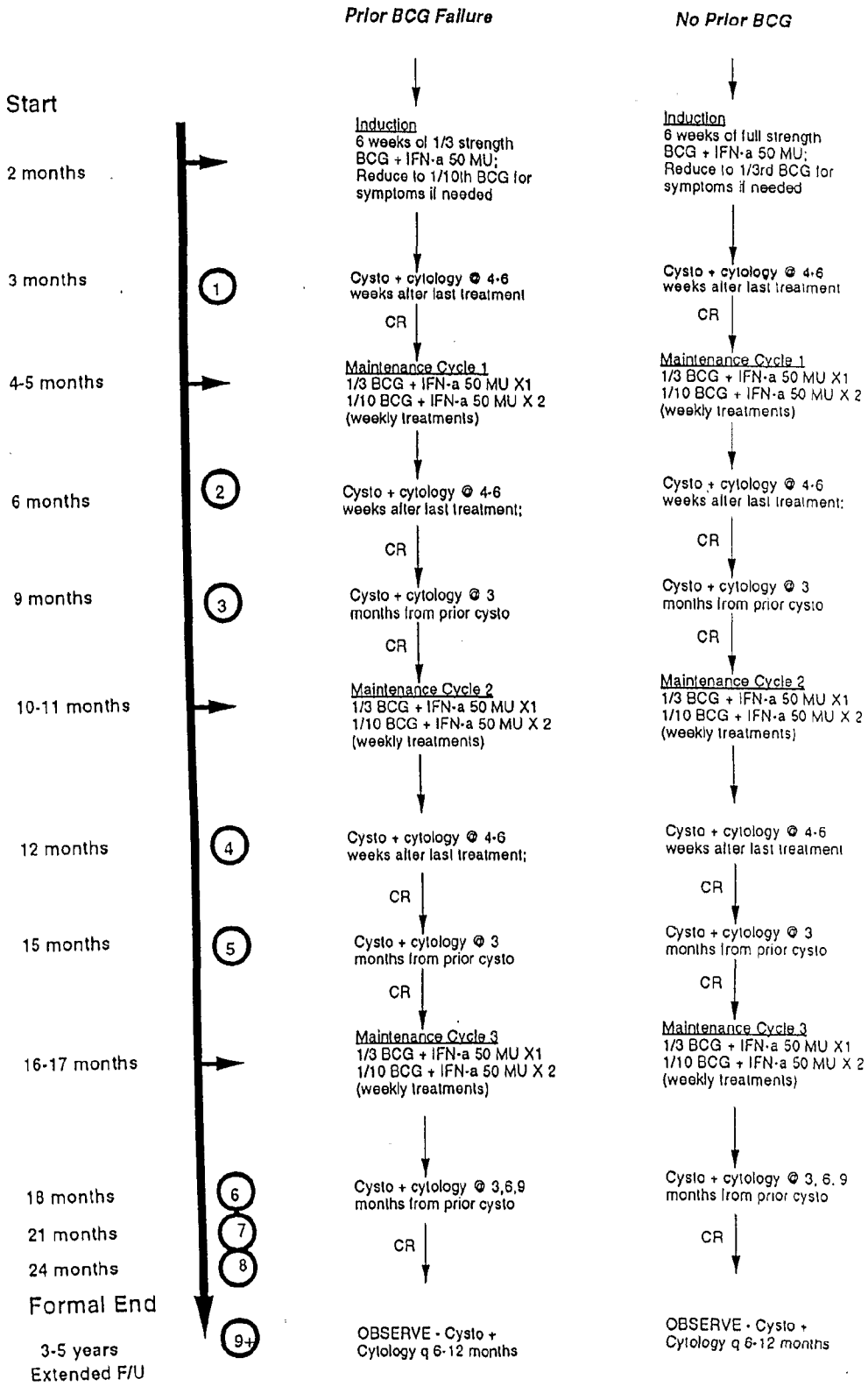
1. Patient Group Induction
   - (i) No prior BCG treatment: BCG + IFN-α (50 MU) X 6-8 wks
   - (ii) BCG Failures: 1/3 dose BCG + IFN-α (50 MU) X 6-8 wks
   - (iii) Relapse: 1/10 BCG + IFN-α (100 MU) X 6 wks

2. Maintenance
   - (i) 3 cycles of 3 at 3, 9, and 15 months after end of induction
   - (ii) Dose 1/3 - 1/10 BCG + IFN-α (50-100 MU)

3. Further Dose Reduction (and Delay) for Intolerance
   - (i) Reduce BCG dose in 1/3 steps (lowest 100th)
   - (ii) Delay extra week for prolonged cystitis
   - (iii) IFN-α dose never reduced

4. Follow-up q 3-4 months by cysto, cytology, biopsy

Schema for BCG + IFN-α Use



The effects of BCG + IFN-α immunotherapeutic treatment on this human "failed BCG patient" category and the naive patient category is shown by Figs. 20-21 and is summarized in Tables E3, E4, E5 and E6 given below.

Table E3

Open Label Study of BCG + IFN-α:
All Patients with Disease-Free Status

Subgroup	No.	12 Mos	24 Mos	Actual
Naive and BCG Failed Patients - Entire Group	60	73%	61%	67%
Non-aggressive	15	87%	61%	67%
Aggressive	45	68%	64%	67%
Primary	11	90%	60%	82%
Recurrent	49	69%	59%	63%
Short duration	44	77%	66%	70%
Long duration	16	60%	45%	56%
BCG Naive	22	89%	52%	77%
BCG Failure	38	61%	56%	58%
Residual papillary TCC (ablation)	7	86% 57%	CR	53%

CR = complete response

Table E4

Open Label Study of BCG + IFN-α Treatment:
Disease Free Status for Prior BCG Failures

Subgroup	No.	12 Mos	24 Mos	Actual
BCG Failed - Entire Group	38	61%	56%	58%
BCG Failed X 1	19	63%	53%	58%
BCG Failed X 2+	19	58%	58%	58%
Non-aggressive	9	67%	53%	56%
Aggressive	29	61%	61%	59%
Minimal-recurrent	15	53%	53%	55%
Multi-recurrent	23	65%	59%	59%
Relapse	5	80%	80%	80%
Refractory	33	58%	51%	55%
Short duration	26	65%	59%	62%
Long duration	12	50%	50%	50%

Table E5

Other Observations: The BCG Failure Group

(i) 12/20 patients (60%) who were told to consider cystectomy are disease free with a normal functioning bladder;

(ii) 13/15 patients (87%) of failures occurred within the first 4 months (at first cystoscopy);

(iii) 4/11 patients (36%) of BCG/IFN failures became disease free with a second course of treatment;

(iv) Patients showing refractory BCG disease (relapse or no response by 6 months) was NOT a poor prognostic indicator;

(v) Patients who were previous BCG failures (> =2) did just as well as those that had failed BCG only once.

Table E6

Practical Clinical Strategies
for Combined Use of Intravesical BCG + IFN-α

(i) The initial therapy for very high-risk patients (e.g., multifocal stage T1 Grade 3, multifocal CIS) is a full dose BCG + IFN-α, 50 MU (million units);

(ii) The salvage therapy for patients who have previously failed BCG is 1/3 dose BCG + IFN-α, 50 MU;

(iii) The salvage therapy for BCG intolerant patients is 1/10 BCG + IFN-α, 100 MU;

(iv) The primary or salvage therapy is effective for both upper tract and superficial transitional cell carcinomas of the bladder.

Experimental Series 4:

A Human Clinical Trial of a Patient

Who Failed BCG Plus Cytokine Therapy Previously

Another clinical evaluation trial performed utilized a different patient category: a human afflicted with superficial bladder cancer whom had previously received BCG plus IFN-α - but nevertheless failed to achieve a disease-free state of meaningful duration. The "failed BCG + a cytokine patient" in this clinical trial presented the following characteristics.

Clinical Hx:

(i) 76 year old male patient ("MN") with Stage T1 Grade 3 TCC in bladder diverticulum s/p transurethral surgery then partial Cystectomy - no cancer found;

(ii) Recurrent Stage T1 Grade 3 at 3 months;

(iii) Treated previously with BCG plus IFN-α X 8 weeks;

(iv) Recurrent multiple Stage T1 Gr 3 plus CIS at 6 weeks post treatment; and refused cystectomy.

This patient MN then underwent a new regimen of BCG plus IL-12 treatment. Specifically, his clinical history was: (a) he received 4 treatments of low-dose BCG (1/3-1/10th dose) plus rhIL-12 intravesically; subsequently (b) his cytology went from positive to atypical after treatment but developed metastasis to liver from a different cancer (likely bowel).

Clinical Changes After BCG Plus IFN-α Treatment

In particular, patient MN responded well to BCG plus IL-12 treatment despite having an inadequate response to BCG + IFNα. This is shown by the in-vitro PBMC response of the patient as shown by Figs. 17A and 22A/B respectively.

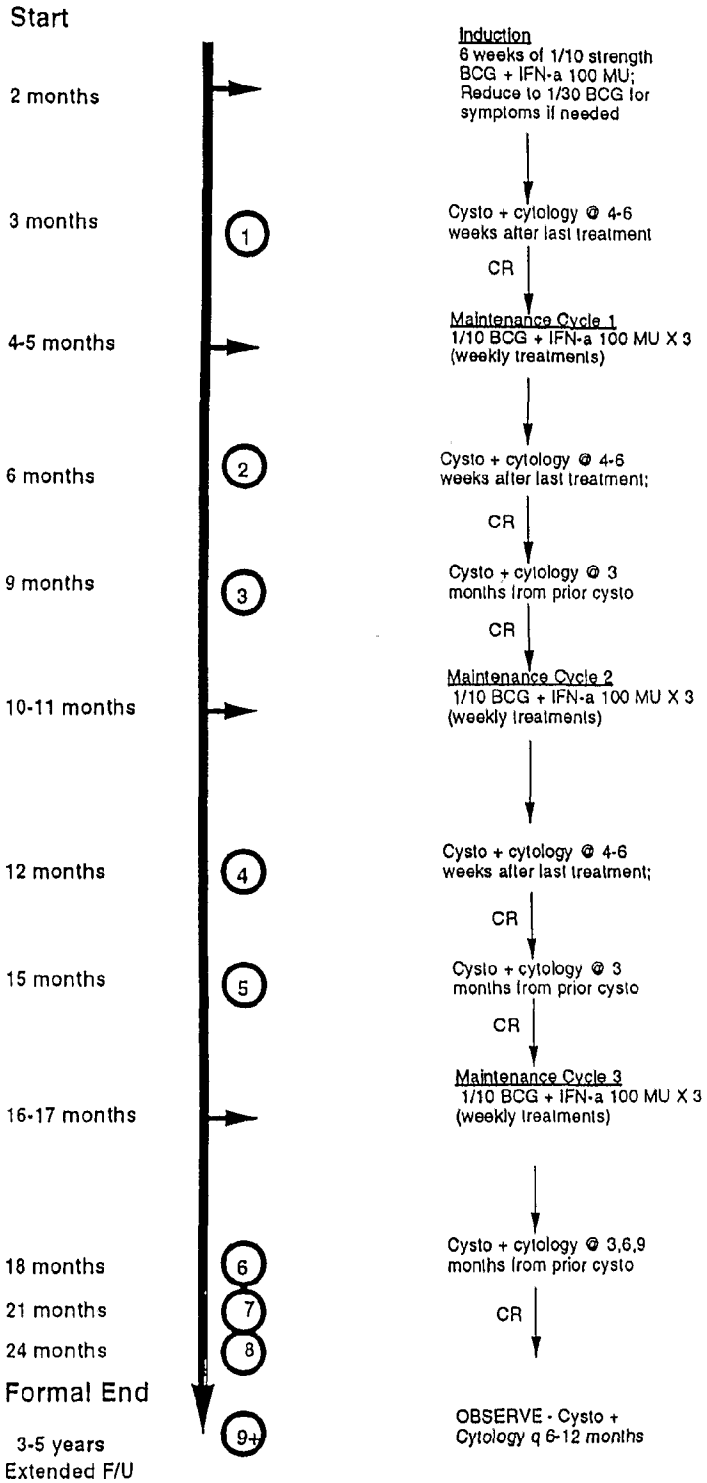
Equally important, patient MN's urinary production of IFN-gamma mass was shown as dramatically increasing as a result of the BCG plus IL-12

immunotherapeutic treatment. This urinary IFN-gamma mass data is shown by Fig. 17B.

Experimental Series 5:

Clinical Evaluation Of Previously Untreated Human Patients With Upper Urinary Tract Cancer

Cancer of the ureter(s) and/or renal pelvis has been difficult to treat to date with conventional immunostimulatory agents and methods. A clinical evaluation of low-dose BCG in combination with 5 human patients having upper tract cancers was therefore undertaken using the treatment scheme shown below.


Start
2 months
3 months
4-5 months
6 months
9 months
10-11 months
12 months
15 months
16-17 months
18 months
21 months
24 months
Formal End
3-5 years
Extended F/U
1
2
3
4
5
6
7
8
9+
Induction
6 weeks of 1/10 strength BCG + IFN-a 100 MU; Reduce to 1/30 BCG for symptoms if needed
Cysto + cytology @ 4-6 weeks after last treatment
CR
Maintenance Cycle 1
1/10 BCG + IFN-a 100 MU X 3 (weekly treatments)
Cysto + cytology @ 4-6 weeks after last treatment;
CR
Cysto + cytology @ 3 months from prior cysto
CR
Maintenance Cycle 2
1/10 BCG + IFN-a 100 MU X 3 (weekly treatments)
Cysto + cytology @ 4-6 weeks after last treatment;
CR
Cysto + cytology @ 3 months from prior cysto
CR
Maintenance Cycle 3
1/10 BCG + IFN-a 100 MU X 3 (weekly treatments)
Cysto + cytology @ 3,6,9 months from prior cysto
CR
OBSERVE - Cysto + Cytology q 6-12 months

Clinical Results

Combination low-dose BCG plus interferon-alpha showed great success in patients with upper tract TCC. Five of 5 patients with a total of 7 upper tracts affected by CIS manifested by positive cytologies have had complete responses to therapy with ongoing remissions at (23+, 20+), 13+, (11+, 6+), 6+ and 5+ months, respectively. And the patient with recurrent low-grade papillary TCC despite laser ablation is disease free after 2 courses of reduced BCG plus interferon-alpha. In all these cases, combination therapy was administered in a retrograde fashion through a temporary, small, atraumatic, 4 French external ureteral stent placed cystoscopically. The smaller size stent permits flow into the uppermost kidney and drainage around the stent to bathe the entire ureter. BCG dose ranged from 1/3, 1/10, 1/30, or 1/100th of the standard bladder dose in 50 cc of physiologic saline combined with 100 MU of IFN-α administered over 2 hours by gravity flow. (Full dose BCG 81 mg/50 ml is too viscous to flow by gravity through a 4 French ureteral stent.) Treatments were administered sequentially usually weekly or biweekly for a total of 6-8 treatments during the induction cycle. Additional maintenance cycles of 3 sequential treatments have also been given to some.

The present invention is not to be restricted in form nor limited in scope except by the claims appended hereto.

What I claim is:

1. An immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer, said patient having failed at least one immunostimulatory therapeutic treatment attempt previously, said immunotherapeutic method comprising the steps of:

choosing the bladder for immunotreatment;

initiating not less than one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one viable Mycobacterium species into the bladder of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain, a substantially non-pathogenic species of Mycobacterium, and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of at least one cytokine in an effective amount in the bladder of the patient, said cytokine being at least one selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and cytokine to act in combination in the bladder as an immunotherapeutic treatment for a preset period of time.

2. An immunotherapeutic method for treating a living patient afflicted with upper urinary tract cancer, said immunotherapeutic method comprising the steps of:

choosing an anatomic site in the upper urinary tract region for immunotreatment;

initiating not less than one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one viable Mycobacterium species to the chosen anatomic site in the upper urinary tract of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain, a substantially non-pathogenic species of Mycobacterium and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of at least one cytokine in an effective amount at the chosen anatomic site in the upper urinary tract of the patient, said cytokine being at least one selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and cytokine to act in combination at the chosen anatomic site in the upper urinary tract as an immunotherapeutic treatment for a preset period of time.

3. An immunotherapeutic method for treating a living patient afflicted with superficial bladder cancer, said patient having failed at least one cytokine-included treatment attempt previously, said immunotherapeutic method comprising the steps of:

identifying the cytokine administered previously to the patient in the failed immunostimulatory treatment;

choosing the bladder for immunotreatment;

initiating not less than one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one viable Mycobacterium species into the bladder of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain, a substantially non-pathogenic species of Mycobacterium and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of not less than one cytokine in an effective amount at the chosen anatomic site in the bladder of the patient, said at least one cytokine being different from that cytokine previously used in the prior failed treatment regimen for the patient, said at least one different cytokine being selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and at least one different cytokine to act in combination at the chosen anatomic site in the bladder as an immunotherapeutic treatment for a preset period of time.

4. The immunotherapeutic treatment as recited in claim 1, 2 or 3 wherein a plurality of cytokines are introduced concurrently.

5. The immunotherapeutic treatment as recited in claim 1, 2 or 3 wherein multiple treatment occasions are given to the patient.

6. A primary immunotherapeutic method for treating a living patient afflicted with a form of urinary cancer, said patient not having received any immunostimulatory agents previously as a cancer treatment regimen, said primary immunotherapeutic method comprising the steps of:

choosing an anatomic site in the body of the patient for immunotreatment;

initiating not less than one treatment occasion for the patient comprised of

(i) introducing an effective quantity of at least one viable Mycobacterium species to the chosen anatomic site in the body of the patient, said Mycobacterium species being one selected from the group consisting of a recombinant DNA Mycobacterial strain, a substantially non-pathogenic species of Mycobacterium and Mycobacterium bovis BCG, and

(ii) causing a concurrent introduction of not less than two cytokines in an effective amount at the chosen anatomic site in the body of the patient, said at least two different cytokines being selected from the group consisting of any type or isoform of interferon-alpha, interferon-beta, interferon-gamma, interleukin-1, interleukin-2, interleukin-3, interleukin-12, interleukin-15, and interleukin-18; and then

allowing said Mycobacterium species and at least two different cytokines to act in combination at the chosen anatomic site in the body as an immunotherapeutic treatment for a preset period of time.

7. The immunotherapeutic method as recited in claim 1, 2, 3, or 6 whereby said Mycobacterium species is present in combination with a blend of 3-9 different cytokines.

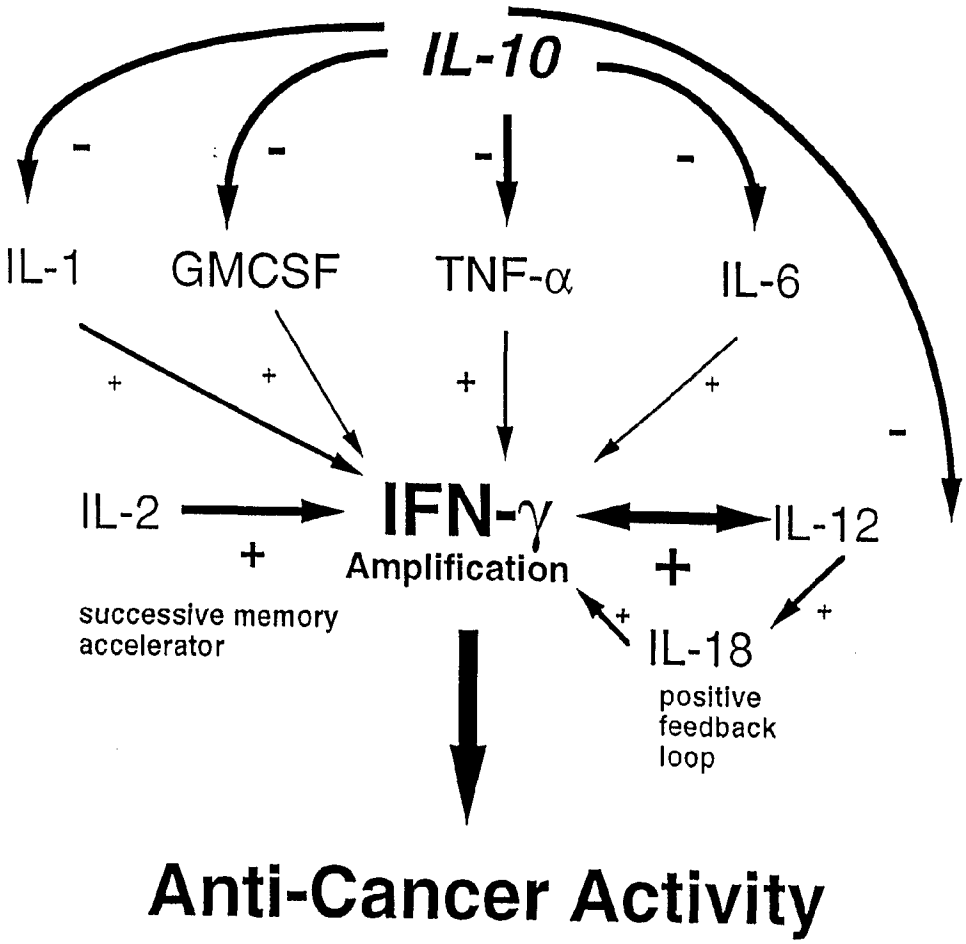

The Cytokine Network Evoked By BCG
IL-10
IL-1
GMCSF
TNF-α
IL-6
IL-2
IFN-γ
Amplification
IL-12
IL-18
successive memory accelerator
positive feedback loop
Anti-Cancer Activity

Fig. 1

Fig. 2A

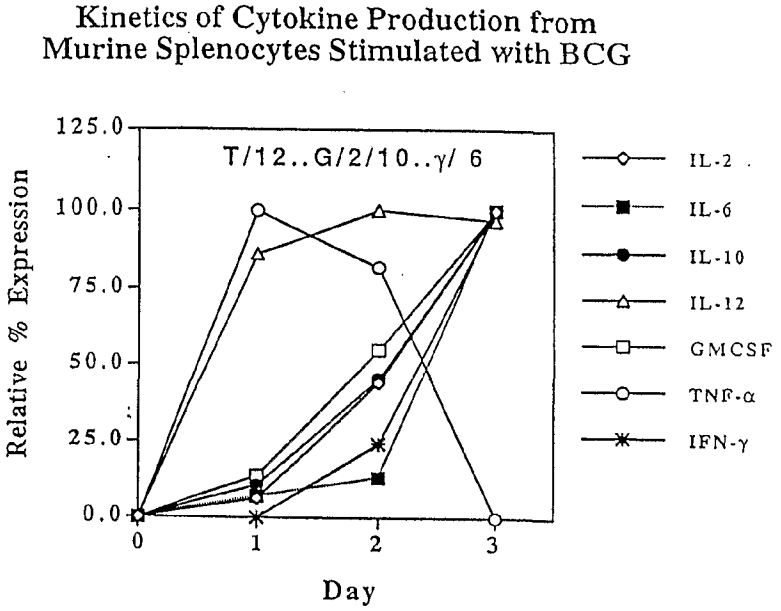

Kinetics of Cytokine Production from
Murine Splenocytes Stimulated with BCG
T/12..G/2/10..γ/ 6
Relative % Expression
125.0
100.0
75.0
50.0
25.0
0.0
0
1
2
3
Day
IL-2
IL-6
IL-10
IL-12
GMCSF
TNF-α
IFN-γ

Fig. 2B

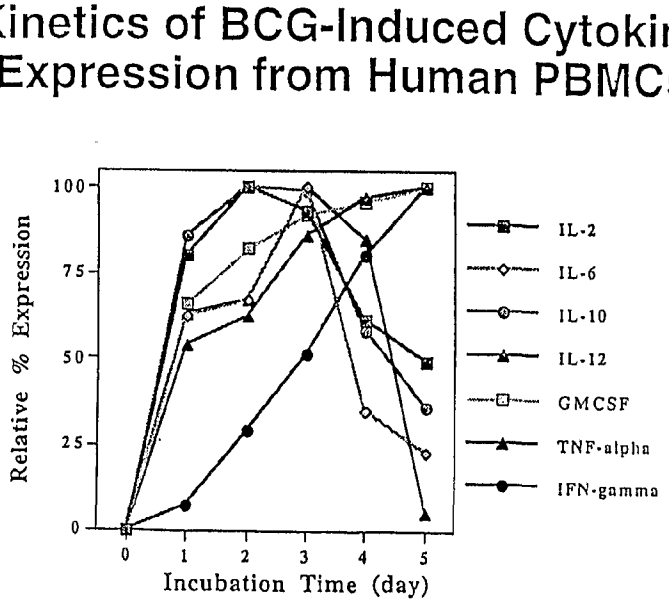

Kinetics of BCG-Induced Cytokine
Expression from Human PBMCs
Relative % Expression
100
75
50
25
0
0
1
2
3
4
5
Incubation Time (day)
IL-2
IL-6
IL-10
IL-12
GMCSF
TNF-alpha
IFN-gamma

Fig. 3A

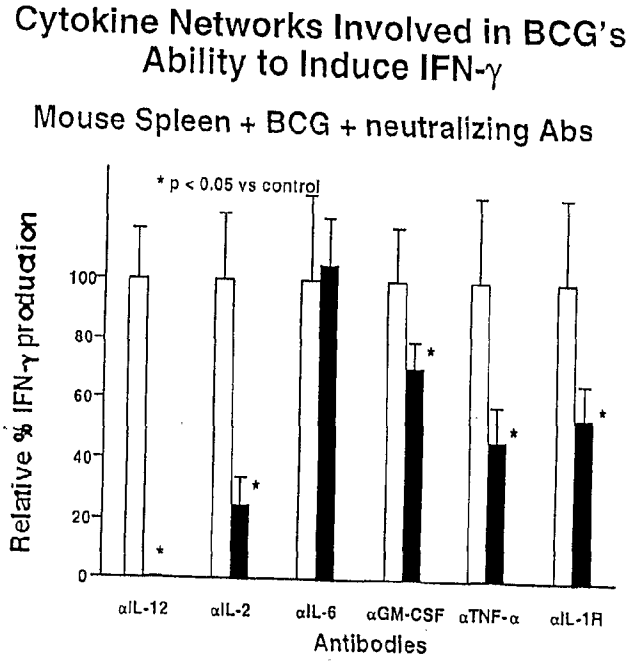

Cytokine Networks Involved in BCG's Ability to Induce IFN-γ
Mouse Spleen + BCG + neutralizing Abs
* p < 0.05 vs control
Relative % IFN-γ production
0
20
40
60
80
100
αIL-12
αIL-2
αIL-6
αGM-CSF
αTNF-α
αIL-1R
Antibodies

Fig. 3B

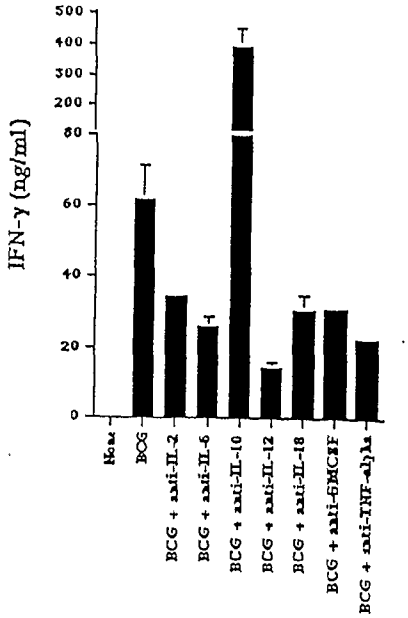
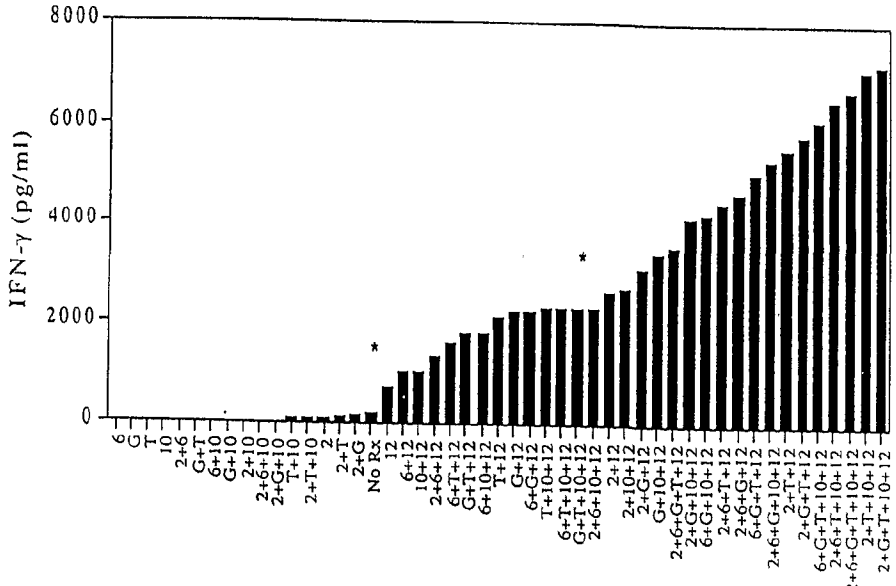

Dependence of BCG-induced IFN-γ from Human PBMCs on Proximal Cytokines
IFN-γ (ng/ml)
0
20
40
60
80
200
300
400
500
Media
BCG
BCG + anti-IL-2
BCG + anti-IL-6
BCG + anti-IL-10
BCG + anti-IL-12
BCG + anti-IL-18
BCG + anti-GMCSF
BCG + anti-TNF-alpha

Fig. 4


Cytokine "Cocktails" Which Mimic BCG Accentuate IFN-γ
IFN-γ (pg/ml)
8000
6000
4000
2000
0
6
G
T
10
2+6
G+T
6+10
G+10
2+10
2+6+10
2+G+10
T+10
2+T+10
2
2+T
2+G
No Rx
12
6+12
10+12
2+6+12
6+T+12
G+T+12
6+10+12
T+12
G+12
6+G+12
T+10+12
6+T+10+12
G+T+10+12
2+6+10+12
2+12
2+10+12
2+G+12
G+10+12
2+6+G+T+12
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2+G+T+12
6+G+T+10+12
2+6+T+10+12
2+6+G+T+10+12
2+T+10+12
2+G+T+10+12

Fig. 5

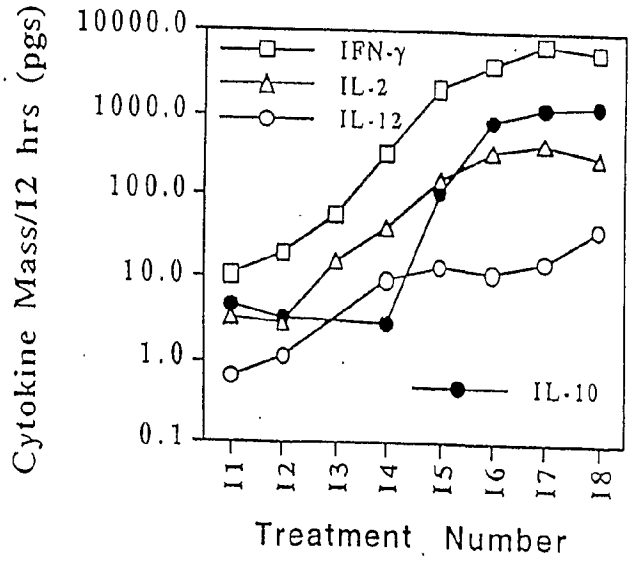
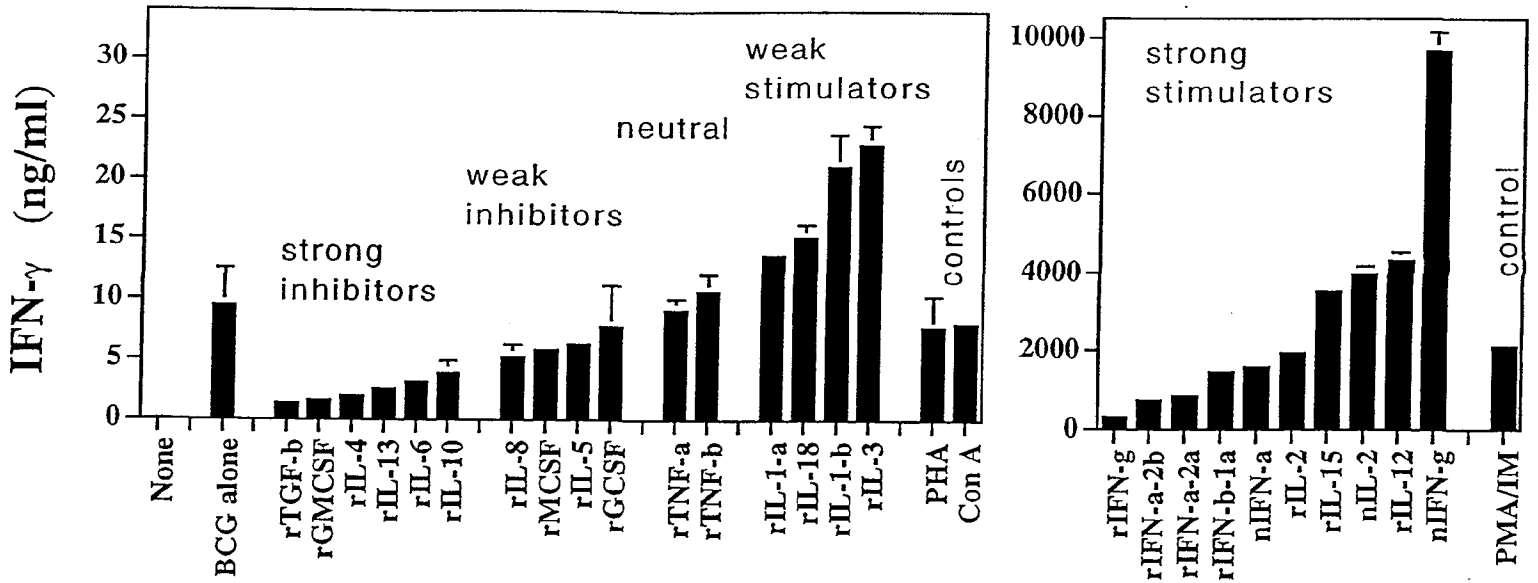

Cytokine Mass/12 hrs (pgs)
10000.0
1000.0
100.0
10.0
1.0
0.1
IFN-γ
IL-2
IL-12
IL-10
I1
I2
I3
I4
I5
I6
I7
I8
Treatment Number

Fig. 6

Effect of Exogenous Recombinant (r) or Natural (n) Cytokines on IFN-γ Production from BCG-Stimulated Human PBMCs



Stimulus: BCG plus Cytokine

Cytokine concentrations: nIL-2 (5RIU/ml); nIFN-g, nIFN-a, rIFN-a-2a/2b, nIFN-a (100 IU/ml ~1-5 ng/ml); all others (1 ng/ml). PHA (5 mcg/ml); Con A (10 mcg/ml); PMA/IM (10 ng & 500 ng/ml)

Fig. 7A

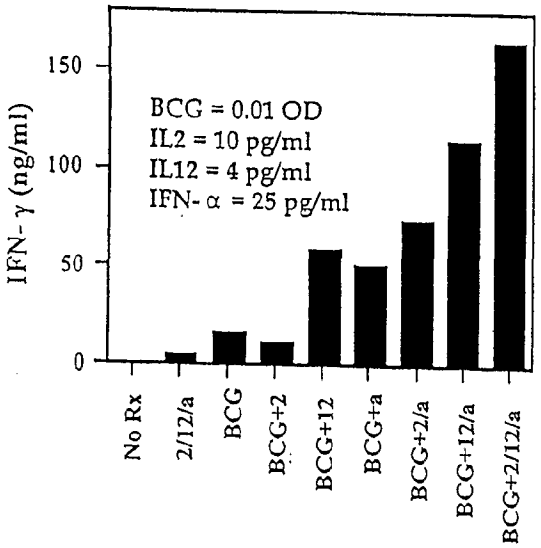

IFN-γ (ng/ml)
150
100
50
0
BCG = 0.01 OD
IL2 = 10 pg/ml
IL12 = 4 pg/ml
IFN-α = 25 pg/ml
No Rx
2/12/a
BCG
BCG+2
BCG+12
BCG+a
BCG+2/a
BCG+12/a
BCG+2/12/a

Fig. 7B

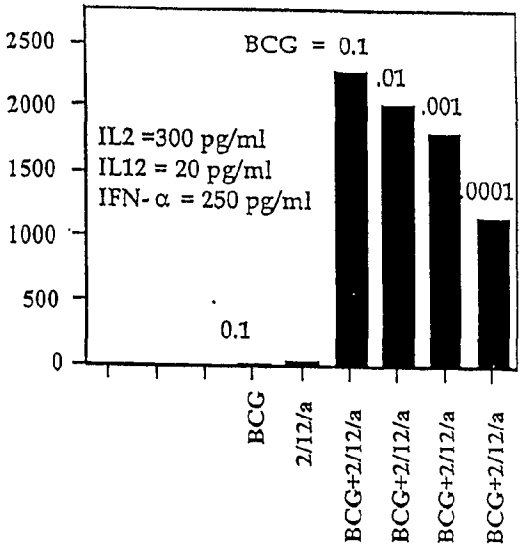
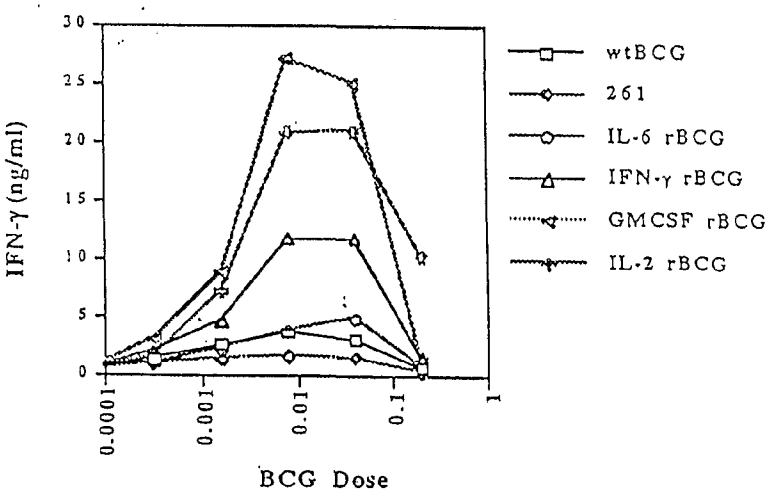

2500
2000
1500
1000
500
0
BCG = 0.1
.01
.001
.0001
IL2 =300 pg/ml
IL12 = 20 pg/ml
IFN-α = 250 pg/ml
0.1
BCG
2/12/a
BCG+2/12/a
BCG+2/12/a
BCG+2/12/a
BCG+2/12/a

Fig. 8


IFN-γ (ng/ml)
30
25
20
15
10
5
0
0.0001
0.001
0.01
0.1
1
BCG Dose
wtBCG
261
IL-6 rBCG
IFN-γ rBCG
GMCSF rBCG
IL-2 rBCG

hIL-2 rBCG + hIFN-a rBCG Provide Synergy in Paracine Fashion

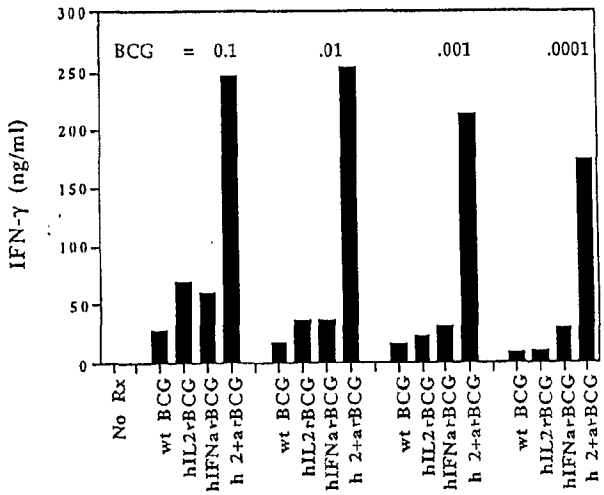


Fig. 9

The Role of IFN-γ in Antitumor Immunity

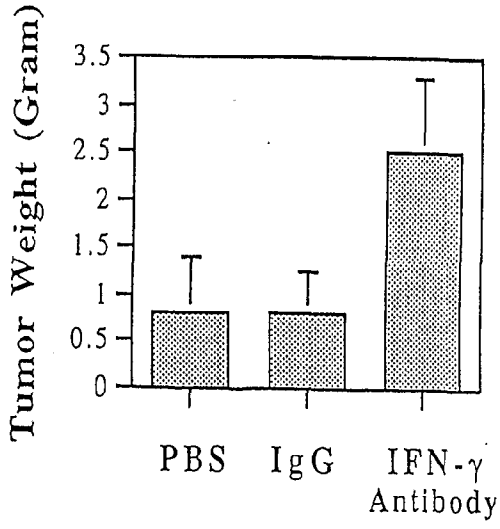


Fig. 10

Fig. 11

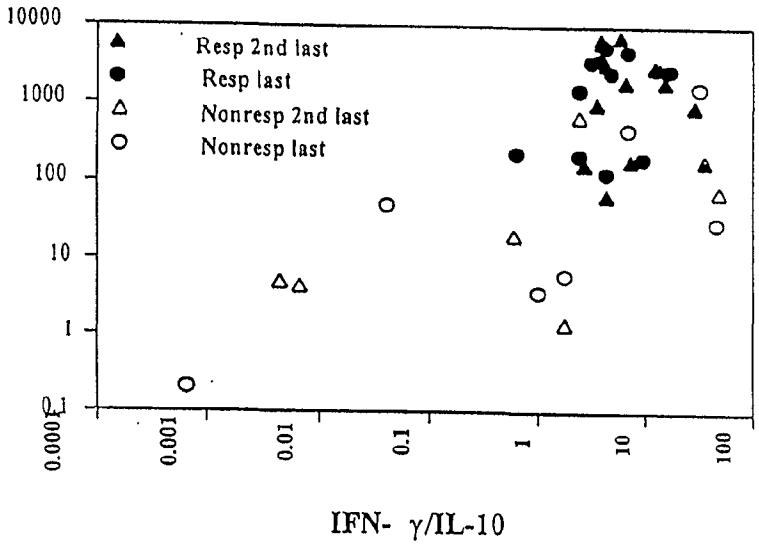

10000
1000
100
10
1
0.1
0.0001
0.001
0.01
0.1
1
10
100
Resp 2nd last
Resp last
Nonresp 2nd last
Nonresp last
IFN- γ/IL-10

Fig. 12

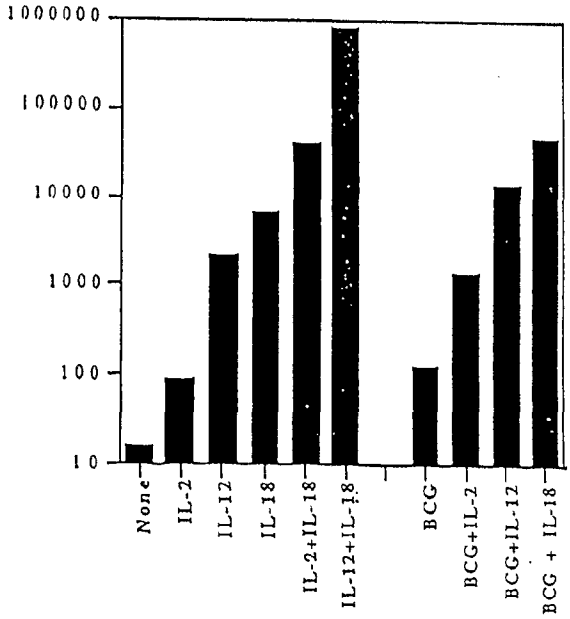

1000000
100000
10000
1000
100
10
None
IL-2
IL-12
IL-18
IL-2+IL-18
IL-12+IL-18
BCG
BCG+IL-2
BCG+IL-12
BCG + IL-18

Fig. 13A

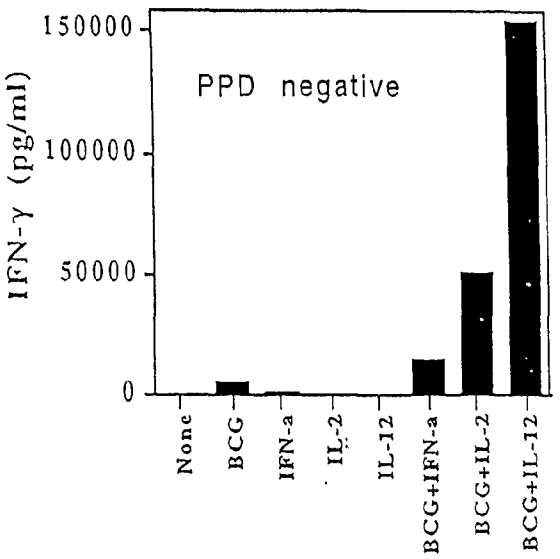
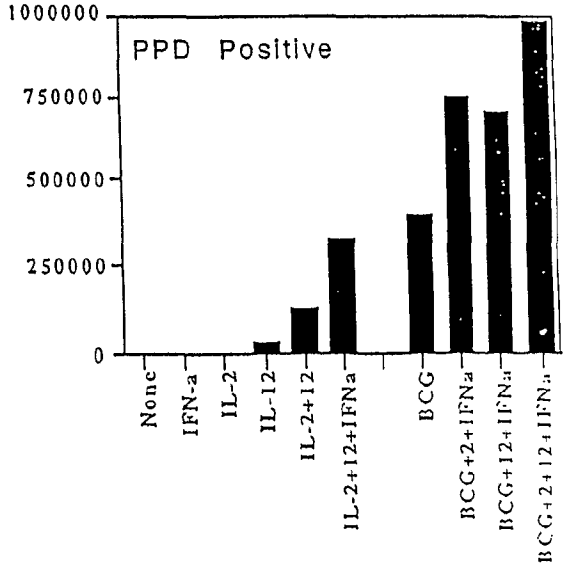


Fig. 13B



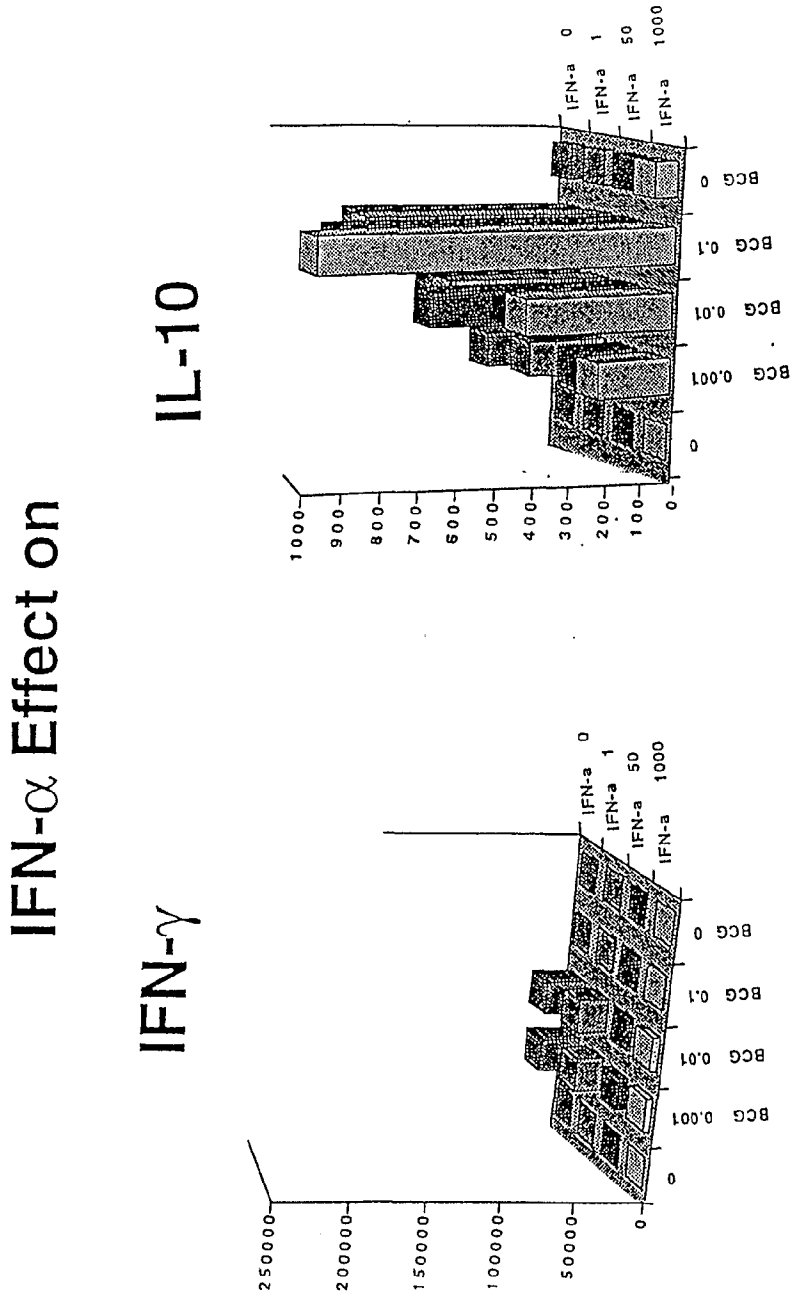


Fig. 14A

Fig. 14B

Fig. 15

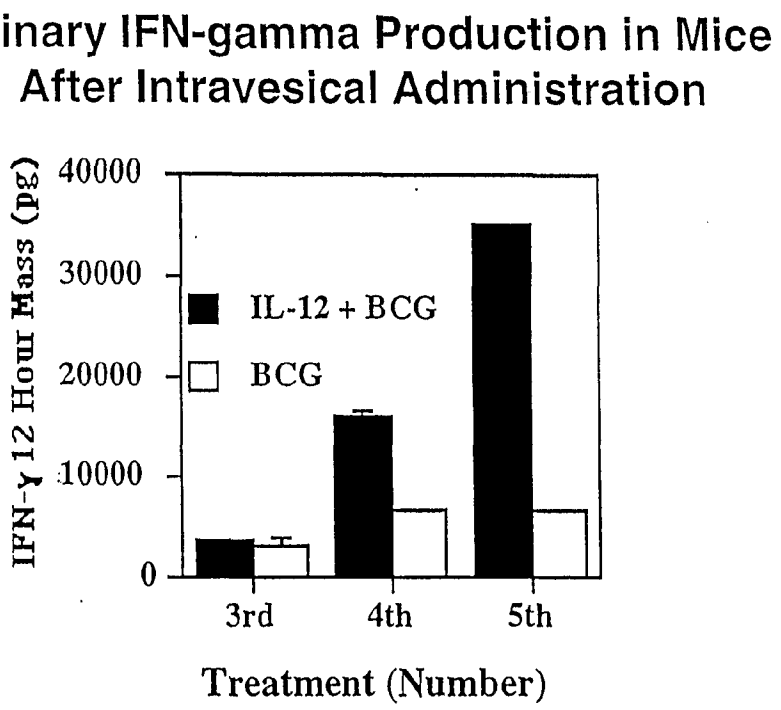
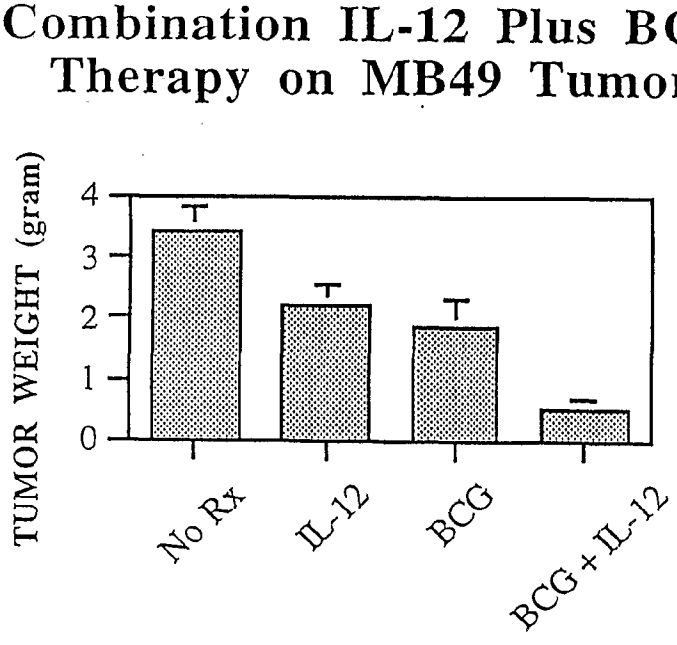

Urinary IFN-gamma Production in Mice After Intravesical Administration
IFN-γ 12 Hour Mass (pg)
40000
30000
20000
10000
0
IL-12 + BCG
BCG
3rd
4th
5th
Treatment (Number)

Fig. 16


Combination IL-12 Plus BCG Therapy on MB49 Tumor
TUMOR WEIGHT (gram)
4
3
2
1
0
No Rx
IL-12
BCG
BCG + IL-12

Treatment Case: BCG plus IL-12

Fig. 17A

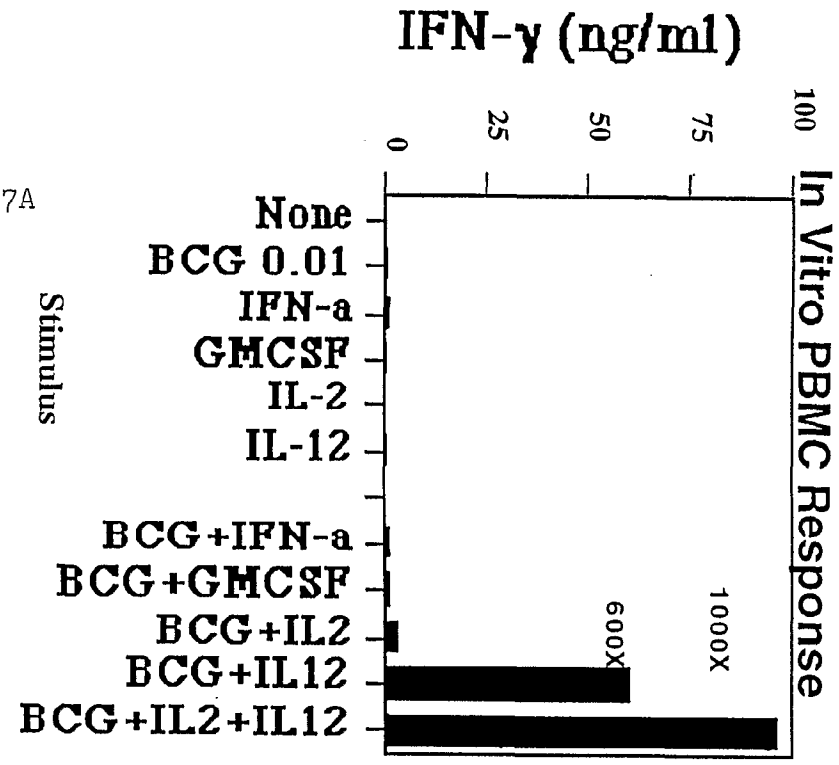


Fig. 17B

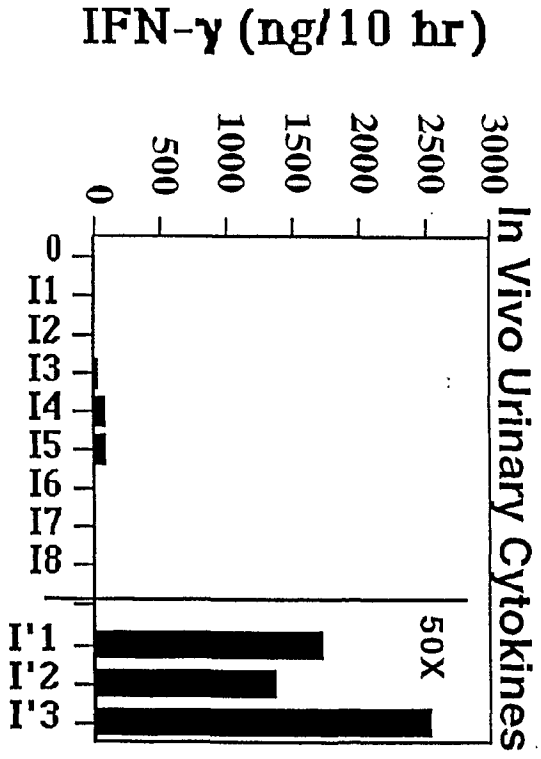


Fig. 18

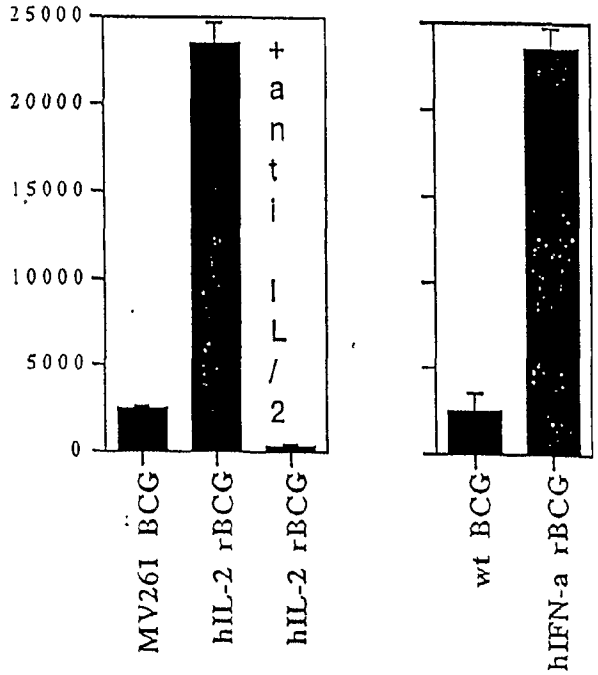

25000
20000
15000
10000
5000
0
anti-IL-2
MV261 BCG
hIL-2 rBCG
hIL-2 rBCG
wt BCG
hIFN-a rBCG

Fig. 19

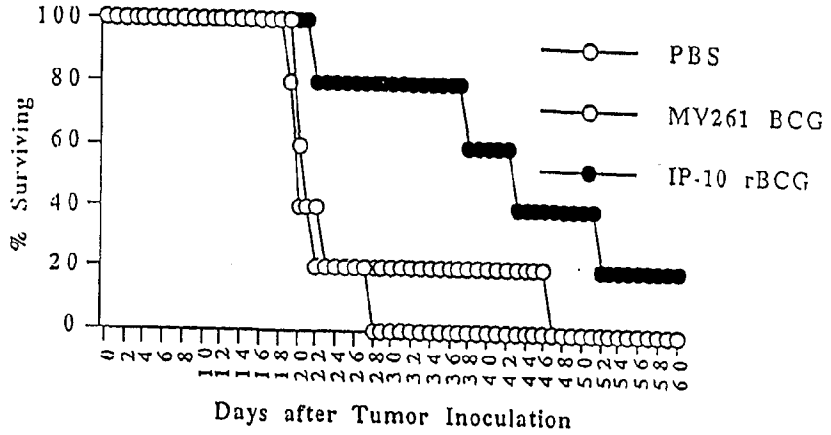

100
80
60
40
20
0
% Surviving
PBS
MV261 BCG
IP-10 rBCG
Days after Tumor Inoculation

Fig. 20

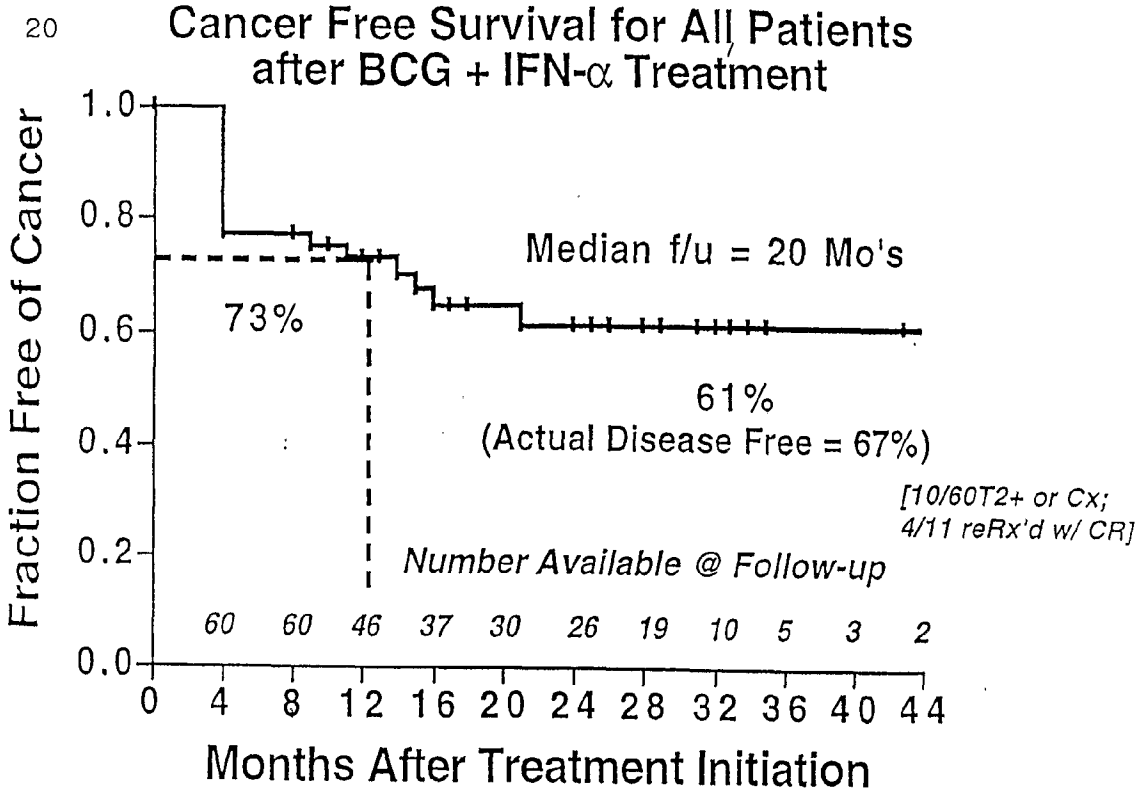

Cancer Free Survival for All Patients
after BCG + IFN-α Treatment
Fraction Free of Cancer
1.0
0.8
0.6
0.4
0.2
0.0
Median f/u = 20 Mo's
73%
61%
(Actual Disease Free = 67%)
[10/60T2+ or Cx;
4/11 reRx'd w/ CR]
Number Available @ Follow-up
60 60 46 37 30 26 19 10 5 3 2
0 4 8 12 16 20 24 28 32 36 40 44
Months After Treatment Initiation

Fig. 21

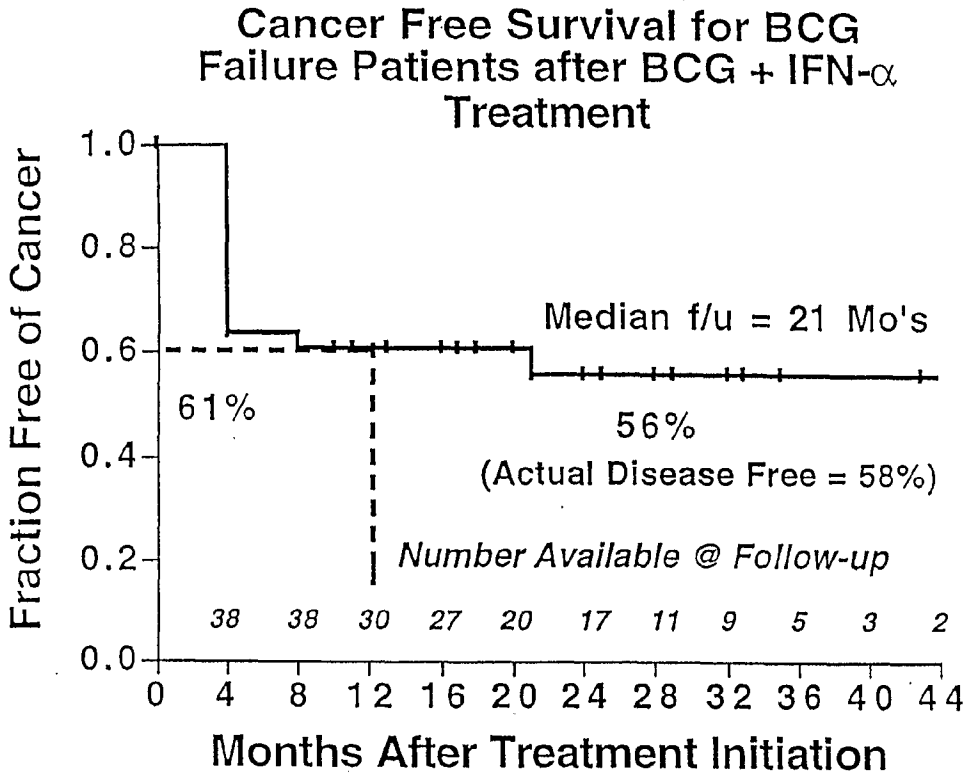

Cancer Free Survival for BCG Failure Patients after BCG + IFN-α Treatment
Fraction Free of Cancer
1.0
0.8
0.6
0.4
0.2
0.0
Median f/u = 21 Mo's
61%
56%
(Actual Disease Free = 58%)
Number Available @ Follow-up
38 38 30 27 20 17 11 9 5 3 2
0 4 8 12 16 20 24 28 32 36 40 44
Months After Treatment Initiation

Fig. 22A

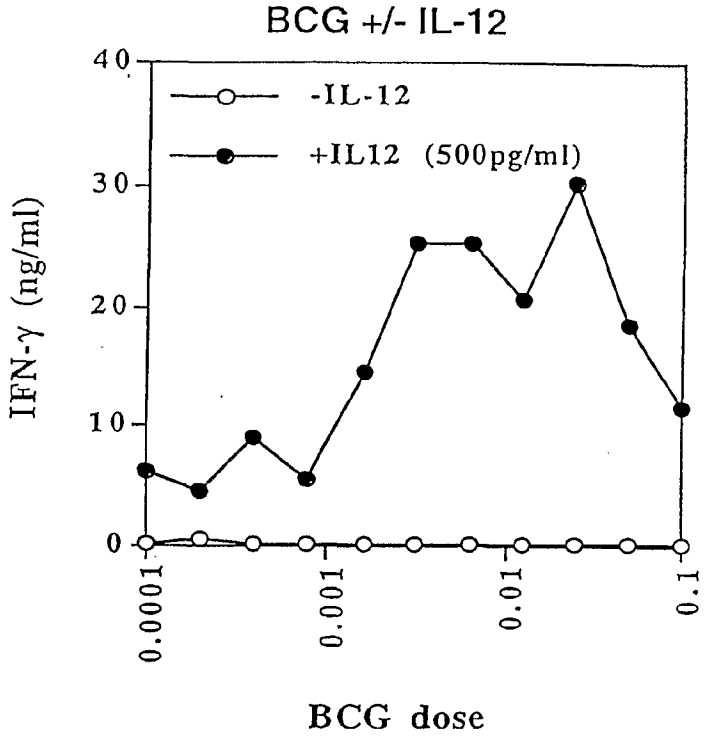
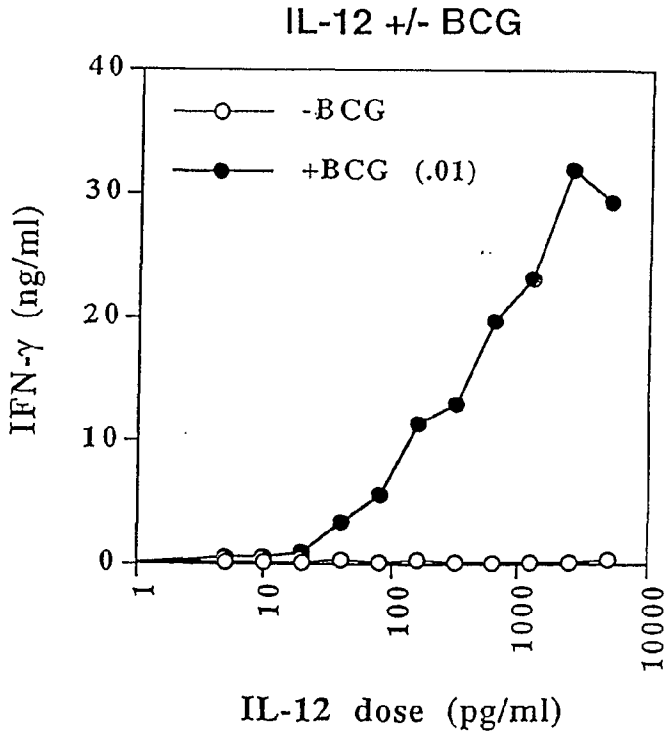


Fig. 22B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/02827

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01N 63/00, 65/00
US CL :424/93.1, 93.2, 200.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.2, 200.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSCIENCE CLUSTER
search terms: BCG, bacillus calmette, urinary tract, bladder, cancer, tumor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COCKETT et al. Bacillus Calmette-Guerin and Interleukin-2 for Treatment of Superficial Bladder Cancer. Journal of Urology. September 1991, Vol. 146, pages 766-770, especially page 768.	1-7
X	BELLDEGRUN et al. Superficial Bladder Cancer: The Role of Interferon-alpha. Journal of Urology. June 1998, Vol. 159, pp. 1793-1801, especially page 1798.	1-7
Y	US 5,830,475 A (ALDOVINI et al.) 03 November 1998, col. 2, line 53, col.1, lines 35-36, col. 2, lines 48-53.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 APRIL 2001	Date of mailing of the international search report 09 MAY 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICIA PATTEN Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/02827

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O'DONNELL et al. Role of IL-12 in the Induction and Potentiation of IFN-gamma in Response to Bacillus Calmette-Guerin. Journal of Immunology. 15 October 1999, Vol. 163, No. 8, pages 4246-4252, especially page 4248.	1-7